

**MICROBIAL METABOLISM OF
ALKYL AND CONDENSED THIOPHENES**

FINAL REPORT

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SUMMARY

Studies on the microbial metabolism of individual organosulfur compounds found in petroleum have been restricted to those compounds that are commercially available. Thus relatively little is known about the metabolites produced from the vast number of organosulfur compounds in petroleum. In collaboration with two chemists who synthesized and provided many petroleum-related organosulfur compounds, this investigation considered the aerobic biotransformations of seven alkyl-substituted thiophenes, six methylbenzothiophenes, four dimethylbenzothiophenes and four methyldibenzothiophenes.

Among the alkylthiophenes, 5-methyl-2-hexadecylthiophene (I), 5-methyl-2-tridecylthiophene (II) and 5-butyl-2-tridecylthiophene (III), 5-methyl-2-(3,7-dimethyloctyl)thiophene (IV), 5-methyl-2-(3,7,11,15-tetramethylhexadecyl)thiophene (V) and 5-ethyl-2-(3,7,11,15-tetramethylhexadecyl)thiophene (VI) were used as substrates in biodegradation studies. The products of their aerobic metabolism by pure bacterial cultures were identified. In most cases, the long alkyl chains of these thiophenes were preferentially attacked and in pure cultures of alkane-degrading bacteria, the major metabolites that accumulated in the medium were 5-methyl-2-thiopheneacetic acid from (I), 5-methyl-2-thiophenecarboxylic acid from (II) and occasionally from (V), 5-butyl-2-thiophenecarboxylic acid from (III) and 5-ethyl-2-thiopheneacetic acid from (VI). These transformations were consistent with the degradation of the alkyl side chains via the beta-oxidation pathway. In contrast, 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid was produced from (IV). Because it was available in greatest supply, (I) was studied most thoroughly. It supported growth of the six *n*-alkane-degrading bacteria tested and (I) was degraded more quickly than pristane but not as quickly as *n*-hexadecane in mixtures of these three compounds. In the presence of Prudhoe Bay crude oil and a mixed culture of petroleum-degrading bacteria, the acid metabolites from (I), (II) and (III) underwent further biotransformations to products that were not detected by the analytical methods used. The addition of *n*-hexadecane to the mixed culture of petroleum-degrading bacteria also enhanced the further biotransformations of the metabolites from (I).

The methyl-substituted benzothiophenes were added to cultures of *Pseudomonas* strain BT1. These compounds would not support growth but were cometabolized when the culture was grown on 1-methylnaphthalene or glucose. Sulfur-containing metabolites were identified by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-Fourier transform infrared spectroscopy (GC-FTIR), and when possible, by comparison with authentic standards. 2,3-Diones were the metabolites from 4-methylbenzothiophene, 5-methylbenzothiophene, 6-methylbenzothiophene and 3,5-dimethylbenzothiophene. The result with the latter compound was unique because the methyl group in the C-3 position was removed to yield 5-methylbenzothiophene-2,3-dione.

Sulfoxides were the major products from 2-methylbenzothiophene, 2,3-dimethylbenzothio-

phene, 2,7-dimethylbenzothiophene and 3,7-dimethylbenzothiophene. Sulfones were also detected as metabolites of the latter four compounds. Cometabolism of 7-methylbenzothiophene yielded the corresponding 2,3-dione, sulfoxide, sulfone and several other unidentified compounds. Cleavage of the thiophene ring of 6-methylbenzothiophene yielded *m*-tolyl methyl sulfoxide. Obviously, the nature of the metabolites produced from these methylbenzothiophenes by isolate BT1 was dependent on the position of the methyl substitution.

The cometabolism of four isomers of methyl-substituted dibenzothiophene was studied using isolate BT1. In addition, the cometabolism of 2-methyldibenzothiophene and 4-methyldibenzothiophene was studied using a newly isolated aromatic-hydrocarbon-degrading bacterium, designated W1. GC-MS and GC-FTIR analyses were used to identify metabolites. In all cases, the corresponding methylbenzothiophene-2,3-dione was produced from each isomer of methyldibenzothiophene. Benzothiophene-2,3-dione was also produced from dibenzothiophene by isolate BT1. Methylhydroxyformylbenzothiophenes were detected as products of the cometabolism of three of the four methyldibenzothiophene isomers. Although a trace amount of benzothiophene-2,3-dione was detected in a culture of BT1 grown in the presence of 2-methyldibenzothiophene, the results obtained in this study clearly showed that the unsubstituted ring of the methyldibenzothiophenes was preferentially cleaved. The prevalence of the 2,3-diones was somewhat unique because among the numerous studies on the microbial metabolism of dibenzothiophene, there has been only one report of benzothiophene-2,3-dione being a metabolite.

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1.0 INTRODUCTION

A recent review of the microbial metabolism of organosulfur compounds in petroleum (Fedorak 1990) demonstrated that very little is known about the metabolism of these compounds. Although hundreds of organic sulfur compounds have been identified in petroleum (Rall *et al.* 1972; Cyr *et al.* 1986; Payzant *et al.* 1986; Sinninghe Damsté *et al.* 1987) fewer than twenty of these have been subjected to biodegradation studies (Fedorak 1990). These studies have been hampered because so few petroleum-related organosulfur compounds are commercially available. Thus little is known about the fate of organosulfur compounds in petroleum-contaminated environments or about the biotransformations of these compounds in petroleum reservoirs. In addition, the use of microorganisms to specifically remove sulfur compounds from fossil fuels has been suggested but only a few model compounds have been used in studies attempting to demonstrate the applicability of this method.

The main objective of this study was to gain a better understanding of the metabolic pathways used by aerobic microorganisms for the biodegradation or biotransformation of organosulfur compounds that are found in petroleum. Two chemists, Dr. J.T. Andersson and Dr. T.M. Peakman, working in Germany synthesized a number of organosulfur compounds that are not commercially available and they supplied these for biotransformation studies. Working with these compounds as substrates, the major focus of this investigation was the identification of intermediates and products of these biotransformations. Specifically, the following three groups of compounds were studied: (a) alkyl-substituted thiophenes, (b) benzothiophene and alkyl-substituted benzothiophenes, and (c) alkyl-substituted dibenzothiophenes. The results provide information relevant to environmental matters, aspects of microbial transformations in petroleum reservoirs and further assessment of the feasibility of biodesulfurization.

2.0 MATERIALS AND METHODS

2.1 Organosulfur compounds

Figure 2.1 shows the seven alkyl thiophenes that Dr. T. M. Peakman supplied and the abbreviations designating each compound. Amounts of 360 to 700 mg of compounds T26, T1-13 and T4-13 were provided. However, much smaller amounts (58 to 100 mg) of the isoprenoidal thiophenes (TMP29, TMP39, TMP40 and TMP41) were supplied. Those compounds provided in the largest quantities were the first to be used in biotransformation studies and were studied most extensively. Later in the study, an additional 620 mg of compound TMP29 were received which allowed more detailed studies of the biotransformation of this compound.

Figure 2.2 shows the structures of the alkyl benzothiophenes that were provided by Dr. J.T.

Andersson. Relatively small amounts of about 500 mg of these compounds were provided. 4-Methylbenzothiophene (4-MBT) and 6-methylbenzothiophene (6-MBT) were supplied as a mixture because the synthesis procedure used gave two isomers (Andersson 1986). However, Dr. Andersson also provided a pure preparation of 4-MBT.

In the original proposal, only six alkyl benzothiophene were to be studied (Figure 2.2). However, our investigations also included the following alkyl benzothiophenes that Dr. Andersson provided: 2,7-dimethylbenzothiophene, 3,5-dimethylbenzothiophene 3,7-dimethylbenzothiophene and 7-ethylbenzothiophene.

The structures of the methyl dibenzothiophenes supplied by Dr. Andersson are shown in Figure 2.3. The synthesis method used yielded a mixture of 1-methyldibenzothiophene and 3-methyldibenzothiophene.

To help verify the identities of some of the metabolites, 5-methylbenzothiophene-2,3-dione and 7-methylbenzothiophene-2,3-dione were synthesized by the methods of Hannoun *et al.* (1982). In addition, the sulfones of 7-MBT, 7-ethylbenzothiophene and 2,7-dimethylbenzothiophene were synthesized according to the method of Bordwell *et al.* (1949). Dr. Andersson provided *m*-tolyl methyl sulfoxide and *m*-tolyl methyl sulfone prepared according to the methods of Cerniani and Modena (1959).

2.2 Cultures and culture conditions

These are described in the individual results sections.

2.3 Silica gel chromatography

To study the decomposition of some sulfoxides in the GC injection port, the polar metabolites in the extracts of cultures grown in the presence of 3-MBT or 2-MBT were isolated by silica gel column chromatography. A slurry of 7 g of silica gel (Fisher Scientific, 100-200 mesh, Type 150A, Grade 644, activated at 125°C for 24 h) in methylene chloride was poured into a glass column (30 cm x 1.1 cm diameter) and the solvent was displaced with 50 mL of *n*-pentane. The methylene chloride extract from the culture was adsorbed to 0.2 g of silica gel, the solvent was removed under a stream of nitrogen gas, and the sample was loaded onto the prepared column which was then developed with 5 mL of *n*-pentane, 5 mL of 20% methylene chloride in *n*-pentane, 25 mL of 50% methylene chloride in *n*-pentane and 30 mL of 50% methanol in benzene. The first 50 mL of the column effluent were discarded and the last 12 mL from the column contained the polar metabolites.

To provide substrates for some mixed culture studies, 2-mL portions of Prudhoe Bay crude oil were fractionated by silica gel chromatography to yield the saturated, aromatic and polar fractions. For these large-scale fractionations, five times the amounts of silica gel and of the solvents listed above were used. The first 65-mL portion of column effluent was discarded because it contained no

components from the crude oil. The next two 60-mL portions contained the saturated and aromatic fractions, respectively. The next 60 mL of effluent were discarded and the final 85 mL to elute contained the polar fraction.

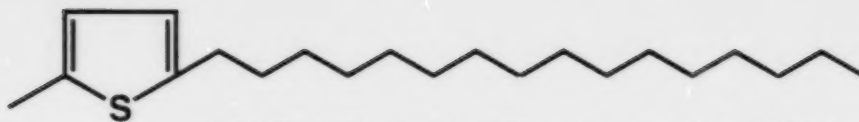
2.4 Analytical methods

After appropriate incubation times, the liquid cultures were acidified with sulfuric acid to pH <2, and extracted with methylene chloride to recover residual substrate and any metabolites that formed. These extracts were analyzed by capillary GC (Fedorak and Grbic'-Galic' 1991) using an instrument that was fitted with a flame ionization detector (FID) and a sulfur-selective, flame photometric detector (FPD) (Fedorak and Westlake 1983a.). The DB-5 capillary column effluent was split so that the sample was analyzed by both detectors simultaneously. This allowed a rapid screening method to determine whether any sulfur-containing metabolites were present.

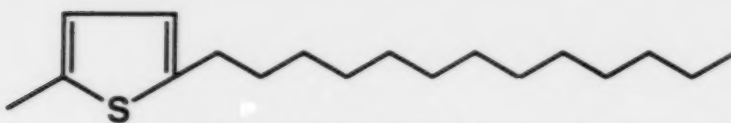
Extracts with sulfur-containing metabolites were analyzed by electron impact GC-MS (Fedorak and Westlake 1986) and GC-FTIR (Fedorak and Grbic'-Galic' 1991) at the analytical service facilities in the Chemistry Department at the University of Alberta. A DB-5 column was used with the GC-MS and an Ultra 2 column (Hewlett-Packard) was used in the GC-FTIR. In addition, a 5970 series mass selective detector (Hewlett-Packard) coupled to a model 5890 GC fitted with a DB-1 column was used for many of the analyses of extracts from cultures grown in the presence of alkyl benzothiophenes or methylthiophenes.

In some cases, samples were analyzed by HPLC using a reverse phase column: 5 mm; 125 by 4 mm; LiChrospher 100 RP-18. The mobile phase was 50:50 acetonitrile:water, the flow rate was 0.5 mL/min or 1.0 mL/min. The effluent was monitored by a UV detector set at 280 nm.

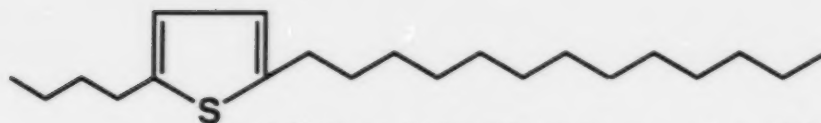
Raney nickel desulfurization and reduction was used to verify the identities of some of the metabolites of some of the alkyl thiophenes. Raney nickel (activity W-2, Aldrich Chemicals) was washed with distilled water, 95% ethanol and anhydrous ethanol as outlined by Furniss et al. (1989). Culture extracts, free of methylene chloride, were refluxed with 0.1 g of Raney nickel in anhydrous ethanol for 4 h. After cooling, the nickel was extracted with dilute alkali (Mozingo *et al.* 1943). Then the extract solution was made acidic with sulfuric acid and extracted with methylene chloride. The GC retention times of the carboxylic acids resulting from the Raney nickel reactions were matched with those of the appropriate authentic standards.



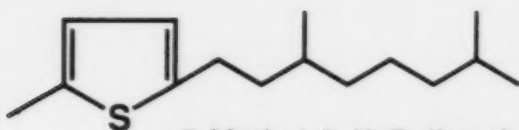
5-Methyl-2-hexadecylthiophene [= T26]



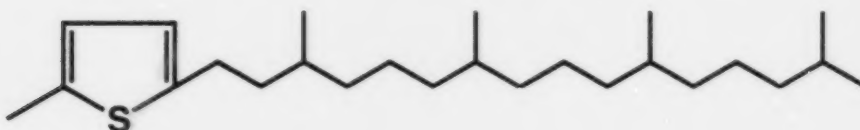
5-Methyl-2-tridecylthiophene [= T1-13]



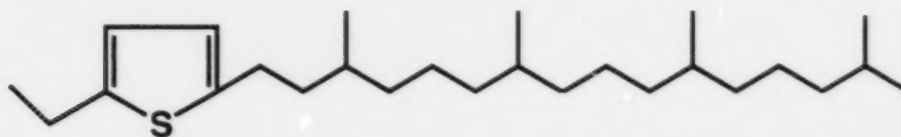
5-Butyl-2-tridecylthiophene [= T4-13]



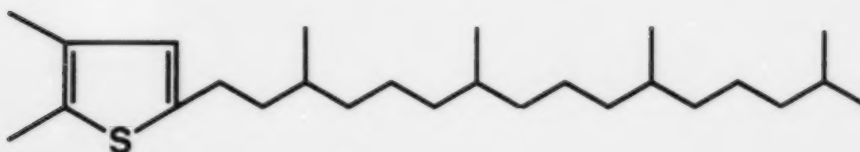
5-Methyl-2-(3,7-dimethyloctyl)thiophene [= TMP29]



5-Methyl-2-(3,7,11,15-tetramethylhexadecyl)thiophene [= TMP39]



5-Ethyl-2-(3,7,11,15-tetramethylhexadecyl)thiophene [= TMP40]



4,5-Dimethyl-2-(3,7,11,15-tetramethylhexadecyl)thiophene [= TMP41]

Figure 2.1. Alkyl thiophenes supplied by Dr. T.M. Peakman for biotransformation studies. Abbreviated designation given in the square brackets.

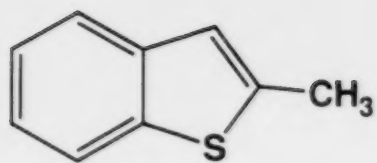
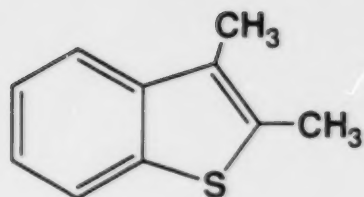
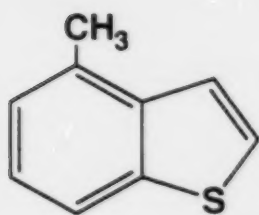
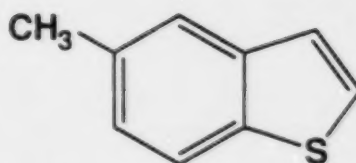
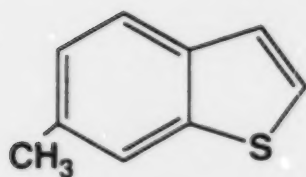
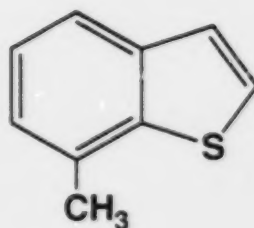
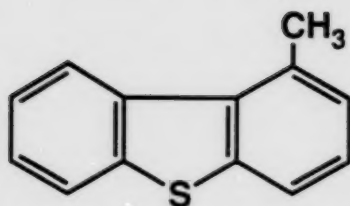
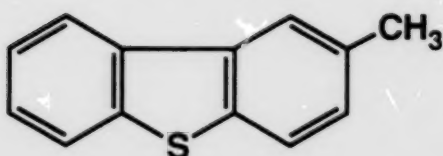
**2-Methyl-****2,3-Dimethyl-****4-Methyl-****5-Methyl-****6-Methyl-****7-Methyl-**

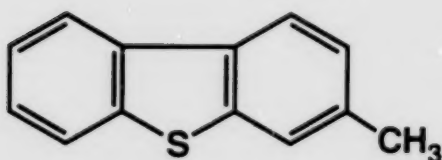
Figure 2.2. Alkyl benzothiophenes originally supplied by Dr. J.T. Andersson for biotransformation studies.



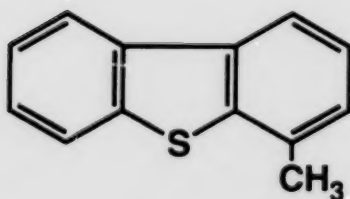
1-Methyl-



2-Methyl-



3-Methyl-



4-Methyl-

Figure 2.3. Alkyl dibenzothiophenes originally supplied by Dr. J.T. Andersson for biotransformation studies.

3.0 RESULTS FROM STUDIES WITH ALKYL THIOPHENES

3.1 *n*-Alkyl-substituted thiophenes as substrates

Seven *n*-alkane-degrading bacterial cultures isolated in our laboratory were used in this study. These strains were designated C1B, C1O, C1Y, C2, C3 (described by Fedorak *et al.* 1988); SE (described by Foght *et al.* 1990); and B+NPA. Individually, the isolates were inoculated into 125-mL flasks containing 50 mL of liquid mineral medium (Fedorak *et al.* 1988) supplemented with trace metals solution (Fedorak and Grbic'-Galic' 1991) and 5 μ g of an *n*-alkyl thiophene as the sole carbon and energy source. The cultures were incubated at 28°C with shaking to provide aeration. This medium was also used to determine whether the isolates could grow on the isoprenoid pristane. All of the isolates, except C3, were capable of degrading pristane.

3.1.1 Studies with 5-methyl-2-hexadecylthiophene (compound T26)

Initial studies focussed on the Gram-positive isolate C2 grown on T26. After 28 days incubation, the culture was acidified, *n*-hexadecane was added as a GC marker and the culture was extracted with methylene chloride. The concentrated extract was analyzed by capillary GC and the FID chromatogram of the extract from isolate C2 is shown in Figure 3.1. From the FPD chromatogram (not shown), each of the major peaks, except *n*-hexadecane, contained sulfur. The last major peak to elute was the substrate T26. The first sulfur-containing peak to elute had the same retention time as a sample of the commercially available compound 5-methyl-2-thiophenecarboxylic acid. When the culture extract and a solution of the 5-methyl-2-thiophenecarboxylic acid were analyzed by GC-MS, the mass spectra of the first sulfur-containing peak in the extract and that of the authentic standard were virtually identical verifying that a metabolite from T26 was 5-methyl-2-thiophenecarboxylic acid.

The mass spectrum of the second sulfur-containing peak to elute from the GC column showed a molecular ion of m/z 156. The base peak for this metabolite was m/z 111 which corresponds to a loss of -COOH. A base peak of m/z 111, resulting from the cleavage beta to the thiophene ring during electron impact mass spectrometry, has been commonly observed by Peakman *et al.* (1989), Peakman and Kock-van Dalen (1990) and Payzant *et al.* (1988). These results suggested that the metabolite was 5-methyl-2-thiopheneacetic acid. This compound is not commercially available to serve as a reference for GC-MS analysis.

A portion of the culture extract from isolate C2 was treated using the method of Fedorak and Westlake (1983b) to produce methyl esters of the carboxylic acids present. In addition to the residual T26 substrate, two sulfur-containing compounds were observed upon GC analysis. The first had the same GC retention time and mass spectrum as methyl ester made from the authentic 5-methyl-2-thiophenecarboxylic acid. GC-MS analysis showed that the methyl ester of the second

metabolite produced a parent ion of m/z 170 with a base peak of m/z 111. These results were consistent with latter metabolite being 5-methyl-2-thiopheneacetic acid.

To verify that the more abundant metabolite was 5-methyl-2-thiopheneacetic acid, combined extracts from cultures of isolate SE grown on compound T26 were desulfurized and reduced with Raney nickel. This reaction was expected to yield heptanoic acid from 5-methyl-2-thiopheneacetic acid. Indeed, the product obtained from the Raney nickel reaction had the identical GC retention time as authentic heptanoic acid.

The other six *n*-alkane-degrading bacterial isolates were also able to use compound T26 as their sole carbon and energy source. Each isolate produced two sulfur-containing metabolites with the same CG retention times as those produced by isolate C2. GC-MS analyses of the extracts from isolates C1Y and SE showed that these metabolites were also 5-methyl-2-thiophenecarboxylic acid and 5-methyl-2-thiopheneacetic acid. The latter metabolite was more abundant than the former metabolite in culture extracts.

These results indicate that the side chain of compound T26 is preferentially attacked and presumably metabolized via a series beta- and/or alpha-oxidations. The fates of the two carboxylic acid metabolites in pure cultures are unknown. We attempted to enrich cultures that will metabolize the two commercially available compounds, 5-methyl-2-thiophenecarboxylic acid and 2-thiopheneacetic acid. The latter compound is similar in structure to 5-methyl-2-thiopheneacetic acid -- the second metabolite. An HPLC method for the analyses of 5-methyl-2-thiophenecarboxylic and 2-thiopheneacetic acid was implemented so that the activities of the enrichment cultures could be monitored. Oil-degrading cultures and activated sludge from the Edmonton sewage treatment plant were used as inocula for these enrichment cultures with either 5-methyl-2-thiophenecarboxylic or 2-thiopheneacetic acid serving as the sole carbon source. However, after several successive transfers, there was no evidence that either of these compounds was metabolized by the enrichment cultures.

The susceptibility of compound T26 to microbial attack was compared to the susceptibilities of *n*-hexadecane and pristane. Numerous studies with crude oil have shown that the *n*-alkanes are degraded before the isoprenoids (Blumer and Sass 1972; Jobson 1974; Fedorak and Westlake 1981). Indeed, the ratio of the amount of *n*-heptadecane to pristane is routinely used to monitor the progress of *n*-alkane degradation in a petroleum-contaminated environment.

In separate experiments, isolates SE and C2 were inoculated into several 500-mL Erlenmeyer flasks that each contained 2 mg of *n*-hexadecane, 2 mg of pristane, 2 mg of compound T26, 1 mg of phenanthrene and 200 mL of mineral medium supplemented with trace metals. Neither isolate can degrade phenanthrene (Fedorak *et al.* 1988; Foght *et al.* 1990) and this aromatic compound was to serve as an internal standard. After various times of incubation at 28°C under shake-flask conditions, one of the replicate cultures was acidified and extracted with methylene chloride. The extract was analyzed by GC to determine the proportions of *n*-hexadecane, pristane and compound T26

remaining relative to phenanthrene. Appropriate sterile controls were incubated with these cultures.

Figure 3.2 shows the results of the time course studies with isolates C2 and SE with the three different substrates. As predicted, both of these isolates degraded hexadecane before pristane. Hexadecane was also preferred over compound T26. However, in both cultures, compound T26 was consumed along with hexadecane and more quickly than pristane.

While monitoring the peak area ratios of the three substrates to phenanthrene in the sterile controls, it was observed that the ratios increased slowly over the incubation time rather than remaining constant as anticipated. This suggested that the phenanthrene was slowly evaporating from the cultures and therefore it was not the ideal compound to choose as an internal standard. In spite of this problem, the interpretations of the data in Figure 3.2 are not altered by the unexpected volatility of phenanthrene.

Subsequent experiments showed that chrysene was a better internal standard because there was no detectable evaporation. Time course studies with isolates C2 and SE incubated with *n*-hexadecane, pristane, compound T26 and chrysene yielded the same results as those shown in Figure 3.2.

3.1.2 Studies with 5-methyl-2-tridecylthiophene (compound T1-13)

Isolates SE and C2 were individually incubated in liquid medium containing compound T1-13 as the sole carbon and energy source. After 2 weeks incubation, these cultures were acidified and extracted with methylene chloride. GC analyses with the FPD showed that isolate SE metabolized virtually all of the substrate leaving one predominant sulfur-containing metabolite. GC-MS analysis and comparison with an authentic standard showed that the product was 5-methyl-2-thiophene-carboxylic acid which would result from six beta-oxidations thereby shortening the side chain by twelve carbon atoms. There was also a trace of another metabolite that was presumably 5-methyl-2-thiopheneacetic acid.

Analysis of the extract from isolate C2 showed two major and one minor sulfur-containing metabolites. GC-MS analysis demonstrated that one of the major metabolites was 5-methyl-2-thiophenecarboxylic acid and that the minor metabolite was 5-methyl-2-thiopheneacetic acid. The parent peak of the other major metabolite was the molecular ion of m/z 168. Other observed ions and some proposed lost fragments are m/z 151 (-OH), 135, 123 (-COOH), 111 and 97 (-CH=CHCOOH). The relative intensities of each of these ions were <40%. These results suggest that the metabolite was 5-methyl-2-thiophenepropenoic acid. The ion at m/z 111 was not abundant. This ion, which results from cleavage beta to the thiophene ring during electron impact mass spectrometry, is usually only abundant when a saturated alkyl chain is substituted onto the methylthiophene moiety. Subsequent enzymatic cleavage of the double bond in 5-methyl-2-thiophenepropenoic acid would yield 5-methyl-2-thiopheneacetic acid after beta-oxidation.

The extract from isolate C2 grown on T1-13 was treated with methanol and sulfuric acid to

produce the methyl esters of the carboxylic acid metabolites. GC-MS analysis showed the presence of a compound with a molecular ion of m/z 182 which was consistent with the metabolite being 5-methyl-2-thiophenepropenoic acid. The base peak was m/z 151 which corresponds to the loss of $-OCH_3$. The next most abundant ion was m/z 123 corresponds to the loss of $-COOCH_3$. Again there was a weak m/z 111 ion which would be expected because of the position of the double bond in this metabolite.

3.1.3 Studies with 5-butyl-2-tridecylthiophene (compound T4-13)

Isolates SE and C2 grew on compound T4-13. GC analysis of the extract from isolate SE showed two sulfur-containing metabolites (Figure 3.4). The mass spectra of these metabolites from CG-MS analysis are shown in Figure 3.5. The more abundant metabolite gave a molecular ion of m/z 184 suggesting that the compound was 5-butyl-2-thiophenecarboxylic acid. The commonly observed cleavage beta to the thiophene ring that occurs during mass spectrometric analysis of alkyl-substituted thiophenes would give the loss of $-CH_2CH_2CH_3$ and a subsequent loss of CO_2 would yield the ions at m/z 141 and 97, respectively. The less abundant metabolite gave a molecular ion of m/z 198 suggesting that the compound was 5-butyl-2-thiopheneacetic acid. Cleavage beta to the thiophene ring would give the the loss of $-CH_2CH_2CH_3$ and a subsequent loss of CH_2 would yield the parent ion of m/z 155 and the ion at m/z 141, respectively.

The extract from isolate SE grown on T4-13 was reacted with methanol and sulfuric acid to yield the methyl esters of the carboxylic acid metabolites. GC-MS analysis showed that the more abundant compound had a molecular ion of m/z 198 and a parent peak at m/z 155. The former ion is consistent with the addition of a methyl group to 5-butyl-2-thiophenecarboxylic acid and the latter ion would arise from the loss of $-CH_2CH_2CH_3$ from the methyl ester via cleavage beta to the thiophene ring during mass spectrometric analysis. The less abundant compound had a molecular ion of m/z 212, a parent peak at m/z 169 and major ion at m/z 153. The molecular ion is consistent with the addition of a methyl group to 5-butyl-2-thiopheneacetic acid and the parent ion would arise from the loss of $-CH_2CH_2CH_3$ from the methyl ester via a cleavage beta to the thiophene ring. The other major ion would result from the loss of $-COOCH_3$.

These results clearly show that the major product of the metabolism of T4-13 is a thiophene carboxylic acid. This is consistent with a series of six beta-oxidations removing six acetate molecules from the C_{13} side chain. 5-Butyl-2-thiopheneacetic acid, which was detected in much lower concentration than 5-butyl-2-thiophenecarboxylic acid, may be the result of an alpha-oxidation. Alternatively, 5-butyl-2-thiopheneacetic acid would result from a series of beta-oxidations of any trace contaminant containing an even number of carbon atoms in the long alkyl side chain that might

be in the T4-13 preparation. These results also suggest that the butyl side chain is too short to be attacked by these pure cultures of *n*-alkane-degrading bacteria and it remained unaltered and attached to the thiophenecarboxylic acid.

3.1.4 Degradation of *n*-alkyl-substituted thiophenes by an oil-degrading enrichment culture with and without Prudhoe Bay crude oil supplementation

The studies described above used alkyl-substituted thiophenes alone, and occasionally mixed with other pure compounds as the carbon source(s) for pure cultures of bacteria. Experiments using mixed bacterial cultures were also done and, in some instances, alkyl thiophenes were added to Prudhoe Bay crude oil and incubated with an oil-degrading culture. The aim of these investigations was to create situations that would more closely mimic conditions that would exist in a petroleum-contaminated environment. That is, the alkyl-substituted thiophenes would be present as part of a complex mixture of compounds in petroleum exposed to a mixed microbial population.

3.1.4.1 Studies with compound T26

In studies with compound T26, 0.2-mL portions of Prudhoe Bay crude oil were added to several 500-mL Erlenmeyer flasks containing 200 mL of mineral medium supplemented with trace metals. Some flasks were supplemented with 2 μ L of T26 and others were supplemented with 2 mg of 5-methyl-2-thiophenecarboxylic acid. Each flask was inoculated with 20 mL of a mixed bacterial culture, designated SLPB, that has been maintained since 1983 by monthly transfers to fresh medium containing Prudhoe Bay crude oil. These cultures were incubated on a rotory shaker at 28°C for 14 days. Appropriate sterile controls were incubated with the test cultures.

After acidification, the Prudhoe Bay oil-containing cultures were extracted with methylene chloride and analyzed by GC. The chromatograms obtained from the FPD are shown in Figure 3.3. The top chromatogram is of the extract from the sterile control and the bottom chromatogram is of the extract from the mixed culture. The mixed culture clearly removed all of the T26. In addition, there was no evidence of the accumulation of any sulfur-containing metabolites such as the 5-methyl-2-thiophenecarboxylic acid or 5-methyl-2-thiopheneacetic acid which were observed to accumulate in the pure cultures (Figure 3.1). Many dibenzothiophenes were evident in the extract from the sterile control but these were absent in the extracts from the oil-degrading mixed culture. The GC analyses of the extracts from the cultures that contained Prudhoe Bay oil and 5-methyl-2-thiophenecarboxylic acid indicated that some of the acid remained after the 2-week incubation (data not shown). Although quantitative results were not obtained from this preliminary trial, it appeared that considerably more 5-methyl-2-thiophenecarboxylic acid remained in the sterile control than in the active cultures. The results shown in Figure 3.3 were the first indications that the metabolites of compound T26 -- 5-methyl-2-thiophenecarboxylic acid and 5-methyl-2-thiopheneacetic

-- acid could be transformed by one of our microbial cultures.

In a subsequent experiment, 0.2-mL portions of Prudhoe Bay crude oil were added to several 500-mL Erlenmeyer flasks containing 200 mL of mineral medium supplemented with trace metals. Some flasks were supplemented with 2 μ L of T26 and others were supplemented with 1 mg of 5-methyl-2-thiophenecarboxylic acid. These flasks were inoculated with 20 mL of the mixed bacterial culture SLPB and they were incubated on a rotory shaker at 28°C for 14 days prior to extraction for GC analysis.

The extraction method was designed to separate the acidic products from the oil. The cultures were adjusted to pH 12 with a NaOH solution and 50 μ L of a benzoic acid solution in methylene chloride was added to give 1 mg of benzoic acid as an internal standard in each culture. Each culture was extracted five times with 15 mL of methylene chloride and these extracts were pooled and analyzed by GC to determine the extent of the petroleum degradation. The aqueous phase was adjusted to pH <2 with sulfuric acid and this was extracted four times with 15 mL of methylene chloride. These extracts, which contained the internal standard and acid metabolites, were combined and analyzed by GC.

Analyses of the extracts from the cultures that contained oil and compound T26 showed no residual T26 and no sulfur-containing acid products. In addition, the extracts from cultures that contained oil and 5-methyl-2-thiophenecarboxylic acid contained none of the latter compound. Sterile controls that contained oil and 1 mg of 5-methyl-2-thiophenecarboxylic acid were also prepared and incubated with the test cultures. The amounts of 5-methyl-2-thiophenecarboxylic acid recovered from sterile controls extracted at the time of inoculation and those extracted after 14 days incubation were virtually identical. To ensure that the extract method could recover sulfur-containing acid products in the presence of biodegraded oil, medium with the mixed bacterial culture SLPB and Prudhoe Bay crude oil were incubated for 14 days to allow oil degradation and then the culture was spiked with 1 mg of 5-methyl-2-thiophenecarboxylic acid just before beginning the extraction procedure. There was no difficulty quantitatively recovering the 5-methyl-2-thiophenecarboxylic acid. Thus these experiments confirmed that in the presence of crude oil, the mixed bacterial culture could degrade T26 and transform the resulting metabolites, one of which was 5-methyl-2-thiophenecarboxylic acid.

When the mixed culture SLPB was grown on compound T26 in the absence of crude oil, a small amount of 5-methyl-2-thiophenecarboxylic acid and a large amount of 5-methyl-2-thiophenecarboxylic acid remained in the medium after 14 days incubation. After a further 14 days incubation, there was virtually no decrease in the amount of the more abundant metabolite. Thus it appears that some component of the crude oil is required to produce further transformations of the metabolites of compound T26.

To determine which components of the crude oil stimulated the degradation of the acidic metabolites of compound T26, Prudhoe Bay crude oil was fractionated into saturated, aromatic and

polar fractions by silica gel chromatography. Portions of these individual fractions and combinations of these fractions were added to medium containing 2 μ L of compound T26 and inoculated with the mixed culture SLPB. Controls included sterile medium containing compound T26 with or without unfractionated Prudhoe Bay oil and cultures containing compound T26 with or without unfractionated Prudhoe Bay oil. After 14 days incubation, the cultures were acidified to pH<2 with sulfuric acid, extracted with methylene chloride and analyzed by GC for residual sulfur compounds.

The data in Table 3.1 summarizes the semi-quantitative results of this experiment. As expected on the basis of previous experiments, (1) no metabolites were found in the sterile controls, (2) with no supplement, a small amount 2-methyl-5-thiophenecarboxylic acid and a larger amount of 2-methyl-5-thiopheneacetic acid remained in the medium, and (3) these acids were not detected when the culture was supplemented with crude oil. Supplementation with the saturated or aromatic fractions, alone or in combination, stimulated the further biotransformation of the acid metabolites.

3.1.4.2 Biotransformation of the metabolites from compound T26 in a culture supplemented with *n*-hexadecane.

Because the addition of the saturated fraction of Prudhoe Bay crude oil stimulated the "degradation" of the acid products from compound T26 by the mixed culture SLPB (Table 3.1), it was speculated that the addition of *n*-hexadecane may have the same effect. To test this hypothesis, eight flasks were prepared, each containing 200 mL of medium and 100 μ L of *n*-hexadecane. Four of these received 2 μ L of compound T26 and the remaining flasks each received 1 mg of 5-methyl-2-thiophenecarboxylic acid. One-half of these flasks was inoculated with the mixed culture SLPB and the others remained as sterile controls. The cultures and controls were acidified and extracted after 15 and 28 days incubation. Just prior to extraction, 1 mg of benzoic acid was added to each flask as an internal standard.

GC analyses showed that by day 15, all of the *n*-hexadecane had been consumed by the mixed cultures. At that time, the culture that contained 5-methyl-2-thiophenecarboxylic acid had 35% as much acid as the corresponding sterile control. By day 28, the other replicate culture contained only 18% as much 5-methyl-2-thiophenecarboxylic acid as the corresponding sterile control.

After 15 days incubation, GC analysis showed that no compound T26 remained in the culture that originally contained this compound. No 5-methyl-2-thiopheneacetic acid was detected in this culture and only a trace amount of 5-methyl-2-thiophenecarboxylic acid was found. By day 28, no 5-methyl-2-thiophenecarboxylic acid was detected. These results are consistent those reported in section 3.1.4.1 and demonstrate that *n*-hexadecane stimulated the further biotransformation the products remaining after the digestion of the hexadecyl side chain.

Table 3.1. Metabolism of compound T26 by the mixed culture SLPB in medium containing various fractions of Prudhoe Bay crude oil.

Inoculum	Supplement	Present in culture extracts		
		T26	5-M-2-TCA ^a	5-M-2-TAA ^b
Sterile	None	+++	-	-
Sterile	Crude oil ^c	+++	-	-
SLPB	None	-	+	+++
SLPB	Crude oil ^c	-	-	-
SLPB	Saturates	-	-	+
SLPB	Aromatics	-	-	++
SLPB	Polars	-	+	+++
SLPB	Saturates & aromatics	-	-	+
SLPB	Aromatics & polars	-	-	+
SLPB	Saturates & polars	-	-	±

^a 5-methyl-2-thiophenecarboxylic acid.

^b 5-methyl-2-thiopheneacetic acid.

^c unfractionated.

3.1.4.3 Studies with compound T1-13 and T4-13

The ability of the mixed culture SLPB to degrade compounds T1-13 and T4-13 in the presence of Prudhoe Bay crude oil was also investigated. In these experiments the cultures inadvertently received 10 μ L of the alkyl-substituted thiophene rather than 2 μ L as was the case with the cultures that received compound T26.

GC analysis of the extract from 14-day-old cultures that originally contained 0.2 mL of Prudhoe Bay crude oil and 10 μ L of compound T1-13 showed extensive oil degradation and the absence of compound T1-13. However, unlike the results obtained with compound T26 and Prudhoe Bay crude oil, the extract from the culture that received compound T1-13 contained a large amount of metabolite 5-methyl-2-thiophenecarboxylic acid. The reason for the accumulation this metabolite is unclear but several hypotheses exist. For example, (1) the high concentration of 5-methyl-2-thiophenecarboxylic acid produced from the metabolism of 10 μ L of compound T1-13 may be inhibitory to the microbes that are able to transform the metabolite; (2) the rate of 5-methyl-2-thiophenecarboxylic acid transformation may be quite slow and analysis after a longer incubation period may show the loss of this metabolite; or (3) the transformation of 5-methyl-2-thiophenecarboxylic acid may be dependent on some component in the crude oil which has been depleted by the microbial culture before all of the 5-methyl-2-thiophenecarboxylic acid was transformed.

GC analysis of the extract from 14-day-old cultures that originally contained 0.2 mL of Prudhoe Bay crude oil and 10 μ L of compound T4-13 showed extensive oil degradation and the absence of compound T4-13. In addition, there was no detectable amount of 5-butyl-2-thiophenecarboxylic acid, the metabolite from compound T4-13 observed in the previous pure culture studies (section 3.1.3). This is the first evidence that 5-butyl-2-thiophenecarboxylic acid could be transformed in one of our microbial cultures.

When the above experiments were repeated with 2 μ L of compound T1-13 or T4-13 rather than 10 μ L of the organosulfur compound, no metabolites were observed in the oil-supplemented mixed cultures after 14 days incubation. These results were consistent with those observed with compound T26 (section 3.1.4.1). 5-Methyl-2-thiophenecarboxylic acid accumulated from compound T1-13 in the mixed cultures without Prudhoe Bay crude oil. Surprisingly, no metabolites from compound T4-13 were observed in the extracts from the SLPB culture grown without Prudhoe Bay crude oil supplementation. This observation was in contrast to those with the pure cultures SE and C2 in which 5-butyl-2-thiophenecarboxylic acid always accumulated in the medium. Clearly the butyl-substituted thiophenecarboxylic acid was susceptible to further biotransformations by the mixed culture in the absence of crude oil, whereas, the methyl-substituted thiophenecarboxylic acid was not.

3.1.5 Degradation of compound T26 by microbes in river water incubated with Prudhoe Bay crude oil

North Saskatchewan River water was collected in Edmonton, upstream of the sewage treatment plant outfall but downstream of several storm sewer outfalls. Two hundred millilitre-portions of the river water were placed in 500-mL Erlenmeyer flasks and these were supplemented with 2 mL of NP solution (Fedorak and Westlake 1981), 2 μ L of compound T26 and 0.2 mL of Prudhoe Bay crude oil. These cultures and suitable sterile controls were incubated at 28°C with shaking. Each week one replicate flask was removed and the culture was acidified and extracted to recover the residual oil for GC analysis.

After seven days incubation, the river water culture had degraded virtually all the *n*-alkanes from the Prudhoe Bay crude oil leaving the isoprenoids, pristane and phytane, as the most abundant peaks in the chromatogram. Also by this time, all of the compound T26 had been degraded and the predominant sulfur-containing compound was 5-methyl-2-thiopheneacetic acid. By day 14, pristane and phytane were still detected in the chromatogram but 5-methyl-2-thiopheneacetic acid was not.

These results showed that the mixed microbial population in the North Saskatchewan River was able to degrade compound T26 and its metabolite, 5-methyl-2-thiopheneacetic acid in the presence of Prudhoe Bay crude oil. In addition, these data showed that compound T26 was more susceptible to degradation than the isoprenoids, pristane and phytane, and this observation was consistent with those obtained with the pure cultures SE and C2 using a mixture of *n*-hexadecane, pristane and compound T26 (section 3.1.1).

3.2 Isoprenoidal thiophenes as substrates.

Most of the studies with these compounds used pure cultures of bacteria that were able to degrade *n*-alkanes and the isoprenoids, pristane and phytane. In addition, because of the small amounts of these materials available, most experiments used a mixture of *n*-tetradecane and the isoprenoidal thiophene of interest. The *n*-alkane served as the primary carbon and energy source in these cultures and transformations of compound TMP29, TMP39 and TMP40 were observed. During the later part of the project, compound TMP29 was provided in sufficient quantity to allow more detailed studies with pure cultures and with mixed microbial cultures (see sections 3.2.5 to 3.2.7).

The isoprenoidal thiophenes were dissolved in ether or methylene chloride and portions of this solution were added to sterile 500-mL Erlenmeyer flasks. After the solvent evaporated, leaving about 5 μ g of thiophene, 200 mL of sterile mineral medium supplemented with trace metals and 10 μ L of tetradecane were added to each flask. These were inoculated with isolate SE or C2 and incubated for 14 days at 28°C with shaking. At the end of the incubation period, the cultures were acidified and extracted with methylene chloride.

3.2.1 Studies with 5-methyl-2-(3,7-dimethyloctyl)thiophene (compound TMP29)

GC analysis of the extract from isolate SE grown on tetradecane in the presence of compound TMP29 showed complete removal of the substituted thiophene and the presence of one sulfur-containing metabolite which had a longer retention time than TMP29. Figure 3.6 shows the mass spectrum of this metabolite with a weak molecular ion at m/z 268. This suggested that the metabolite was 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid. Cleavage beta to the thiophene ring via a McClafferty rearrangement (McClafferty 1980) would give the base peak of m/z 142 which can subsequently lose CO_2 to give the ion at m/z 98. In competition with this fragmentation pathway is the normal beta-cleavage to give m/z 141 which can also lose CO_2 to give, in this case, m/z 97 which is the second most abundant ion in the mass spectrum. Fragmentation of the branched alkyl chain would produce the ions m/z 43, 57, 71 and 85 (Silverstein and Bassler 1967). These correspond to the fragments C_3H_7 , C_4H_9 , C_5H_{11} , and C_6H_{13} , respectively.

The methyl ester of the metabolite formed by isolate SE grown on tetradecane in the presence of compound TMP29 was synthesized and analyzed by GC-MS. The mass spectrum of the methyl ester was consistent with the metabolite being 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid. For example, the molecular ion and the parent peak were m/z 282 and 256, respectively. The latter ion would result from cleavage beta to the thiophene ring and a McClafferty rearrangement. These two ions were each 14 mass units greater than those observed for the free acid providing more evidence that the carboxyl group was on the thiophene ring. The second most abundant ion was m/z 97 which would arise from the loss of $-\text{COOCH}_3$ from the parent peak. In addition, peaks at m/z 43, 57 and 71 were observed resulting from the fragmentation of the branched alkyl chain.

Isolate C2 was grown for 14 days on pristane in the presence of compound TMP29. GC analysis of the extract showed a peak with the same retention time as 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid. The methyl ester of this metabolite was produced by acid-catalyzed methylation and this was analyzed by GC-MS. The mass spectrum of the methylated product was the same as that described in the preceding paragraph.

The oxidation of the methyl group of compound TMP29 by the pure cultures of strains SE and C2 giving 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid was unique among the compounds examined in this study. With each of the other alkyl thiophenes, the longer side chain was oxidized and the methyl group was unaltered. Even the butyl group of compound T4-13 remained unaltered in pure cultures studies. The results with compound TMP29 demonstrate that its branched side chain is quite resistant to microbial attack.

3.2.2 Studies with 5-methyl-2-(3,7,11,15-tetramethylhexadecyl)thiophene (compound TMP39)

In several experiments, cultures were incubated for 14 days in the presence of *n*-tetradecane and compound TMP39. GC analyses of the culture-extracts showed little or no compound TMP39 remained and no sulfur-containing metabolites were detected. However, in one experiment, the extract from a culture of SE yielded a sulfur-containing metabolite with the same GC retention time as 5-methyl-2-thiophenecarboxylic acid.

3.2.3 Studies with 5-ethyl-2-(3,7,11,15-tetramethylhexadecyl)thiophene (compound TMP40)

GC analysis of the extract from isolate SE grown on tetradecane in the presence of compound TMP40 showed virtually complete removal of the substituted thiophene and the presence of one sulfur-containing metabolite. The mass spectrum of the metabolite is shown in Figure 3.7 and it suggests that the compound is 5-ethyl-2-thiopheneacetic acid. The molecular ion is m/z 170 and the base peak at m/z 125 would result from the loss of $-COOH$ due to cleavage beta to the thiophene ring. The base peak of compound TMP40 is also m/z 125 (data not shown). These results show that the 20-carbon branched side chain of TMP40 was attacked by isolate SE yielding 5-ethyl-2-thiopheneacetic acid which accumulated in the medium in this pure culture study.

3.2.4 Studies with 4,5-dimethyl-2-(3,7,11,15-tetramethylhexadecyl)thiophene (compound TMP41)

Compound TMP41 was used in only one experiment in which isolates SE and C2 were grown on *n*-tetradecane in the presence of TMP41. GC analysis of the extract from isolate SE showed that little of the substituted thiophene remained in the culture. In contrast, much of compound TMP41 remained in the medium in which isolate C2 was grown. A predominant sulfur-containing compound eluting at 24.95 min was found in the culture extracts but not in the sterile control. GC-MS analysis of the extract from the C2 culture showed that this compound was dibenzothiophene! The source of this contaminant is unknown. No metabolites were detected in these extracts, and further work is required to determine the fate of compound TMP41 in these pure cultures.

3.2.5 Degradation of compound TMP29 by an oil-degrading enrichment culture with and without Prudhoe Bay crude oil supplementation

Several 500-mL Erlenmeyer flasks containing 200 mL of mineral medium supplemented with trace metals and 2 μ L of TMP29 were inoculated with 20 mL of the mixed bacterial culture SLPB. Some flasks were supplemented with 0.2 mL of Prudhoe Bay crude oil. These cultures and appropriate sterile controls were incubated on a rotary shaker at 28°C for 14 days prior to extraction

for GC analysis.

The extracts from the cultures that received crude oil contained no compound TMP29 after the 2-week incubation period. Nor was 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid, the metabolite observed in pure culture studies with compound TMP29, or any other sulfur-containing metabolite detected. In contrast, compound TMP29 remained undegraded in the cultures that received no crude oil. Thus the presence of the crude oil in this mixed culture stimulated the degradation of compound TMP29.

3.2.6 Degradation of compound TMP29 by microbes in river water incubated with Prudhoe Bay crude oil

Cultures were set up in the same manner as described in section 3.1.5 with 2 μ L of compound TMP29 added in place of compound T26. GC analysis of the culture extracts showed that after 7 days incubation, the culture had degraded essentially all the *n*-alkanes from the Prudhoe Bay crude oil leaving the isoprenoids, pristane and phytane, as the most abundant peaks in the chromatogram. Compound TMP29 remained in the chromatogram and no sulfur-containing metabolite was detected. However, after 14 days of incubation, neither of these isoprenoid alkanes nor compound TMP29 were observed in the culture extract. Nor was 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid, the metabolite observed in pure culture studies with compound TMP29, detected. Indeed, no sulfur-containing metabolites were observed in the culture after 14 days incubation.

These results clearly showed that the mixed microbial population in the North Saskatchewan River was able to degrade compound TMP29 and its metabolite in the presence of Prudhoe Bay crude oil. In addition, these data showed that compound TMP29 was less susceptible to degradation than the *n*-alkanes but about as susceptible as the isoprenoids, pristane and phytane.

3.2.7 Metabolism of compound TMP29 by *n*-alkane-degrading pure bacterial cultures with Prudhoe Bay crude oil supplementation

Isolates C2 and SE were inoculated individually into 500-mL Erlenmeyer flasks containing 200 mL of mineral medium supplemented with trace metals and 2 μ L of compound TMP29. Each flask was supplemented with 0.2 mL of Prudhoe Bay crude oil and these cultures and appropriate sterile controls were incubated on a rotory shaker at 28°C. After 14 days incubation, half of the replicate cultures were extracted to separate the acidic products from the oil, as outlined in section 3.1.4.1. The remaining cultures were extracted after a further 14 days incubation.

After 14 days incubation, all of the *n*-alkanes had been degraded, but pristane and phytane remained. These isopenoidal hydrocarbons also remained after 28 days incubation. GC-FPD analyses of the extracts containing the acidic products from these cultures showed the presence of a novel, sulfur-containing metabolite which eluted after 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid and

gave a very broad peak. The extract from the C2 culture was treated to yield methyl esters that were analyzed by GC-MS. Peaks with mass spectra corresponding to the methyl esters of 5-methyl-2-thiopheneacetic acid and 8-(5-carboxy-2-thiophenyl)-2,6-dimethyloctanoic acid were observed. The mass spectrum of the newly observed compound is shown in Figure 3.8. The weak molecular ion at m/z 326 was consistent with the compound being the dimethyl ester of 8-(5-carboxy-2-thiophenyl)-2,6-dimethyloctanoic acid. Chemical ionization GC-MS with ammonia yielded a $(M+1)^+$ ion at m/z 327, confirming the molecular weight of 326. Cleavage beta to the thiophene ring would yield the base peak m/z 155 (Figure 3.8).

Pirnik *et al.* (1974) found that omega-oxidation yielding dicarboxylic acid intermediates plays a key role in the metabolism of the isoprenoid pristane. The detection of 8-(5-carboxy-2-thiophenyl)-2,6-dimethyloctanoic acid is the first evidence of omega-oxidation of the isoprenoidal thiophenes. However, in the pure culture studies with isolates SE and C2, this dicarboxylic acid persisted over the 28-day incubation period.

3.3 Summary

The initial survey with compound T26 showed that each of the six *n*-alkane-degrading bacterial isolates grew on this compounds. Subsequent studies demonstrated that the long side chains of the three *n*-alkylthiophenes were preferentially attacked and metabolized, presumably, via a series of beta-oxidations. Metabolism of compound T26, with a C_{16} side chain, yielded predominantly 5-methyl-2-thiopheneacetic acid whereas metabolism of compounds T1-13 and T4-13, with C_{13} side chains, yielded predominantly 5-methyl-2-thiophenecarboxylic acid and 5-butyl-2-thiophene-carboxylic acid, respectively. Interestingly, the butyl side chain of compound T4-13 remained unaltered indicating that it was too short to be attacked by these pure cultures of *n*-alkane-degrading bacteria.

Small amounts of 5-methyl-2-thiophenecarboxylic acid and 5-butyl-2-thiopheneacetic acid were detected in cultures grown on compounds T26 and T4-13, respectively. These products would not arise via beta-oxidations of the substrates but they might result from the alpha-oxidation (oxidative decarboxylation) (Ratledge 1978) of one of the predominant metabolites. Alternatively, these may be the products of the beta-oxidations of trace contaminants in the preparations of compounds T26 and T4-13. For example, GC-MS analysis of the preparation of compound T26 showed the presence of a contaminant with a molecular ion at m/z 308 and a base peak at m/z 111. These data suggest that the contaminant was 2-methyl-5-pentadecylthiophene which would yield 5-methyl-2-thiophenecarboxylic acid after a series of seven beta-oxidations.

The accumulations of carboxylic acids in the culture media observed during this study were similar to results of other investigations of the microbial metabolism of cyclic compounds with long alkyl side chains. For example, Davis and Raymond (1961) found that phenylacetic acid accumulated

in cultures of various strains of *Nocardia* sp. grown on dodecylbenzene. Similarly, studies with *n*-alkane-degrading bacteria and fungi showed that the predominant metabolites from 2-undecyltetrahydrothiophene and 2-dodecyltetrahydrothiophene were 2-tetrahydrothiophenecarboxylic acid and 2-tetrahydrothiopheneacetic acid, respectively, and that these metabolites persisted in the culture media (Fedorak *et al.* 1988). Other studies have shown that the resulting carboxylic acids were transient intermediates. These include work with *Nocardia salomonicolor* (Sariaslani *et al.* 1974) and several fungi (Fedorak and Westlake 1986) grown on dodecylbenzene. In each of the above mentioned studies, the initial microbial attack was on the alkyl side chain rather than on the cyclic moiety.

As was observed with the *n*-alkylthiophenes, the long side chains of two of the isoprenoidal thiophenes, compounds TMP29 and TMP39, were metabolized by pure cultures to give 5-methyl-2-thiophenecarboxylic acid and 5-ethyl-2-thiopheneacetic acid, respectively. This likely occurred via a terminal oxidation of the tetramethylhexadecyl moiety and subsequent beta-oxidations removing C₃- (propionyl-SCoA) and C₂-units (acetyl-SCoA) as was observed during the bacterial metabolism of pristane (McKenna and Kallio 1971; Pirnik *et al.* 1974). However, the formation of 5-ethyl-2-thiopheneacetic acid from compound TMP40 indicates that removal of the isoprenoidal side chain can occur without omega-oxidation. The inability to find metabolites from compound TMP39 in several of our experiments may indicate that omega-oxidation plays a role in its biodegradation. Dicarboxylic acids arising from this type of oxidation would be too polar to be detected by our analytical methods. Further investigations are required to determine whether any dicarboxylic acids were produced.

The oxidation of the methyl group of compound TMP29 by strain SE giving 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid was unique among the compounds examined in this study. With each of the other alkyl thiophenes, the longer side chain was oxidized and the methyl group was unaltered. The results with compound TMP29 suggest that its dimethyloctyl side chain was quite resistant to microbial attack.

When added to Prudhoe Bay crude oil, each of the three *n*-alkylthiophenes was metabolized by the oil-degrading mixed culture SLPB. Indeed, the presence of the crude oil enhanced the degradation of compounds T26 and T1-13 because no carboxylic acid intermediates were detected in the extracts from the mixed culture supplemented with crude oil. Also, when 5-methyl-2-thiophenecarboxylic acid was added to oil-containing medium with the SLPB culture, this acid could not be detected after 14 days incubation. However, the mixed culture did not transform the metabolites from compounds T26 and T1-13 in the absence of crude oil in the medium. In contrast, the SLPB culture did transform 5-butyl-2-thiophenecarboxylic acid produced from compound T4-13 when there was no crude oil in the medium. Supplementing the SLPB culture with the saturated and/or aromatic fractions of Prudhoe Bay crude oil or with *n*-hexadecane stimulated the further biotransformation of 5-methyl-2-thiophene-

carboxylic acid and 5-methyl-2-thiopheneacetic acid.

The biotransformation products of the carboxylic acid intermediates of the alkylthiophenes have not been determined. However, several studies have shown that bacterial isolates can grow on various thiophene carboxylic acids. For example, Cripps (1973) isolated a bacterium that could use 2-thiophenecarboxylic acid as its sole carbon, sulfur and energy source and Kanagawa and Kelly (1987) isolated strains of *Rhodococcus* that used 2-thiophenecarboxylic acid, 5-methyl-2-thiophenecarboxylic acid and 2-thiopheneacetic acid as sole carbon and energy sources. Similarly, Evans and Venables (1990) isolated a *Vibrio* species that could grow on 2-thiophenecarboxylic acid and 2-thiopheneacetic acid. Oxygen uptake experiments showed that resting cells of the *Vibrio* species were able to oxidize several thiophene derivatives including 2-methylthiophene, 3-thiophenecarboxylic acid, and 3-thiopheneacetic acid. Whether the carboxylic acid intermediates of the alkylthiophenes served as growth substrates for some of the bacteria in the SLPB mixed culture or whether these intermediates were cometabolized to products that were not detected by our analytical method remains to be determined.

This work showed that five of the seven model alkylthiophenes studied were readily metabolized by pure cultures of *n*-alkane-degrading bacteria that removed the side chains leaving carboxylic acid intermediates. The exceptions were compound TMP41, for which we were unable to detect metabolites, and compound TMP29 for which the methyl group was preferentially oxidized. In addition, compound TMP29 yielded 8-(5-carboxy-2-thiophenyl)-2,6-dimethyloctanoic acid in a pure culture that had been supplemented with crude oil. This indicated that diterminatal (or omega) oxidation occurred.

When added to a crude oil, the *n*-alkylthiophenes were metabolized by a mixed culture of petroleum-degrading microorganisms and the carboxylic acid intermediates were further transformed to unidentified products. Similarly, in the presence of crude oil, compound TMP29 was metabolized by mixed cultures and no sulfur-containing metabolites were found. Although these alkylthiophenes are usually minor components of petroleum, they are quite susceptible to microbial metabolism and therefore should not accumulate in petroleum-contaminated environments.

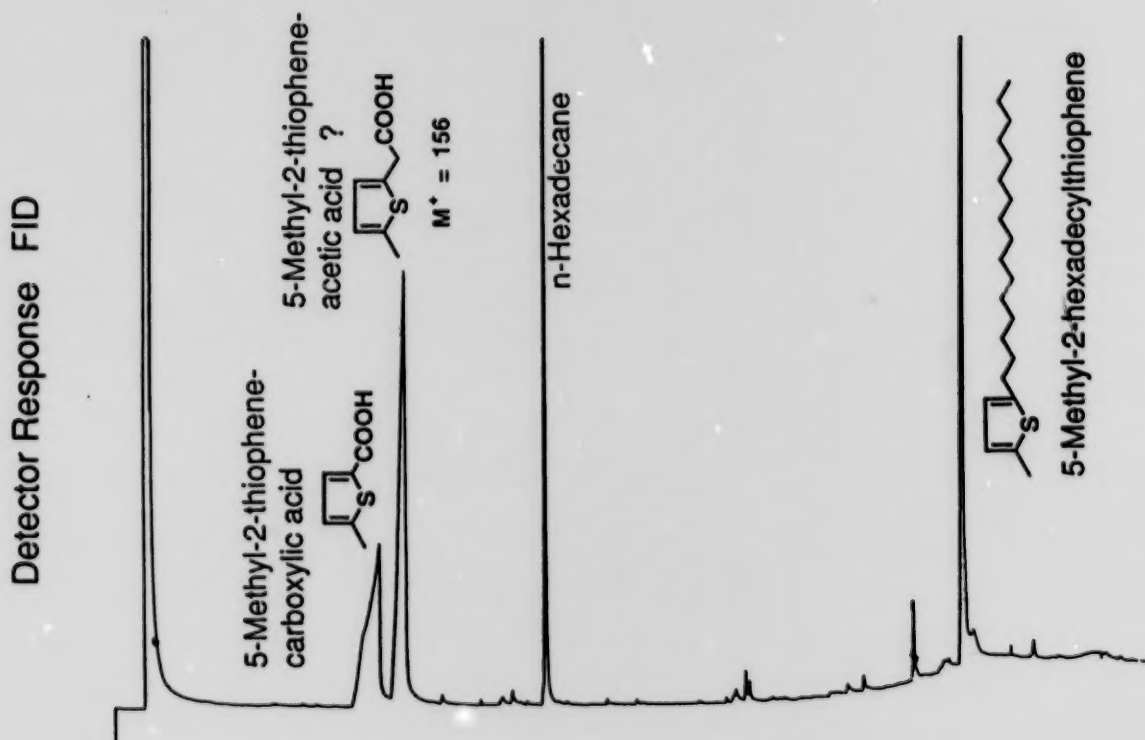


Figure 3.1. GC analysis of the methylene chloride extract of isolate C2 grown on compound T26.

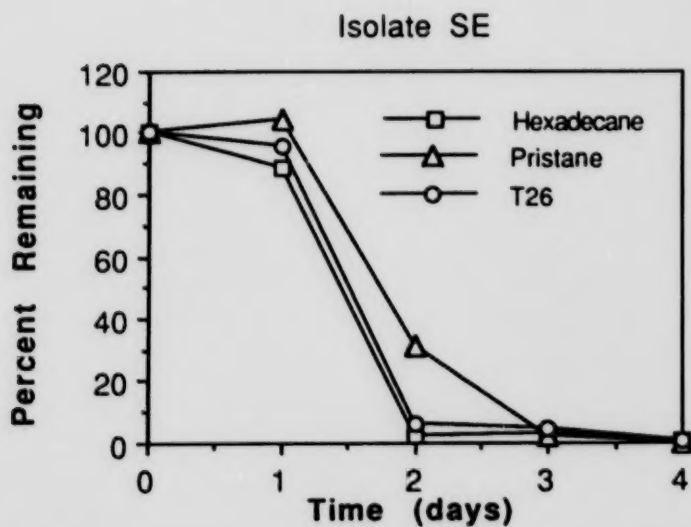
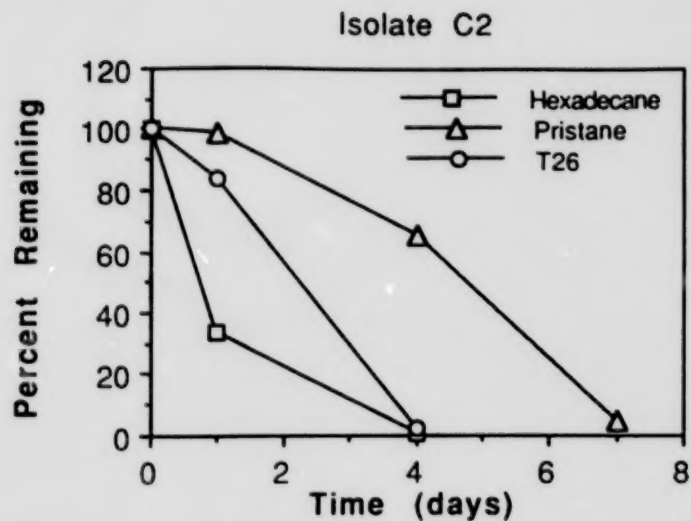


Figure 3.2. Comparisons of the relative susceptibilities of hexadecane, pristane and compound T26 to biodegradation by bacterial isolates C2 and SE.

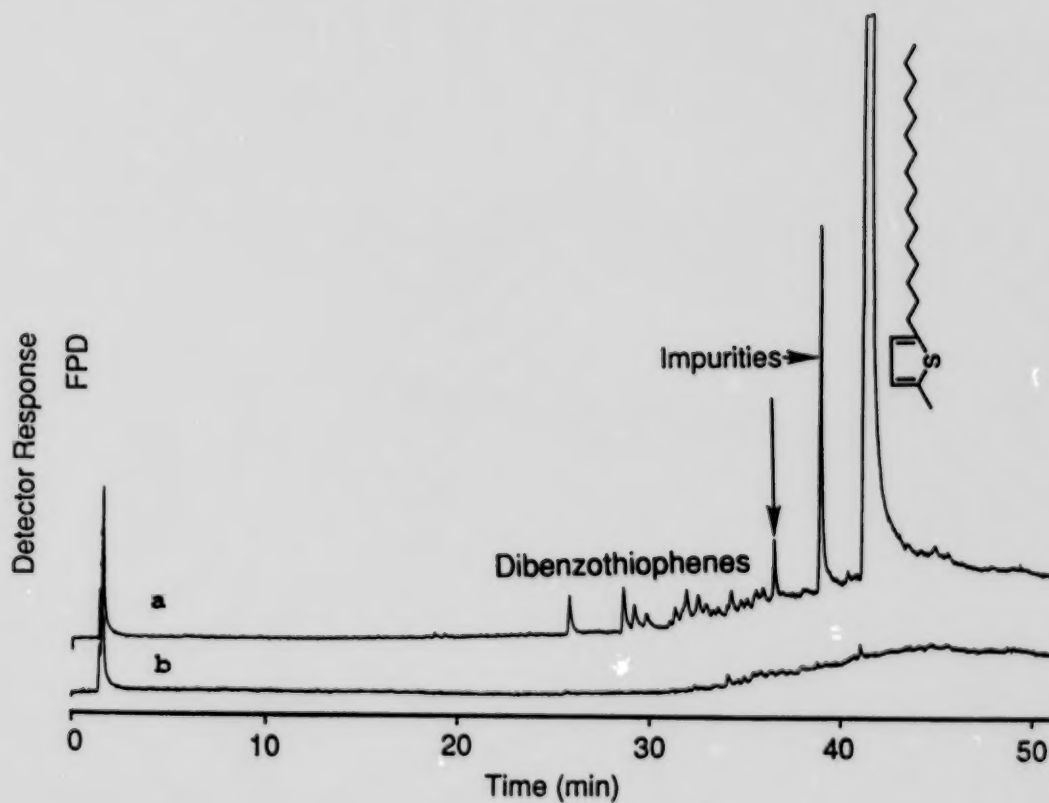


Figure 3.3. GC analyses of the methylene chloride extracts from (a) a sterile control containing Prudhoe Bay crude oil supplement with compound T26 and (b) an oil-degrading mixed culture growing on Prudhoe Bay crude oil supplement with compound T26. Both samples were extracted after 14 days incubation.

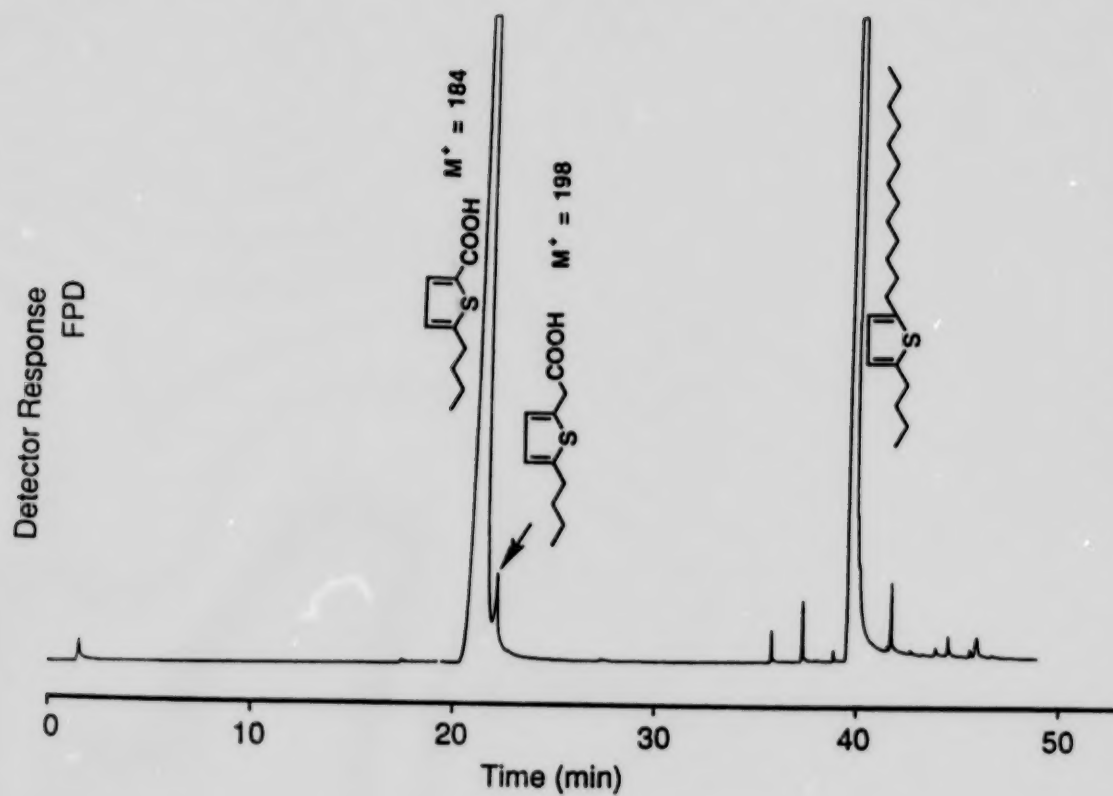


Figure 3.4. GC analysis of the methylene chloride extract of isolate SE grown on compound T4-13.

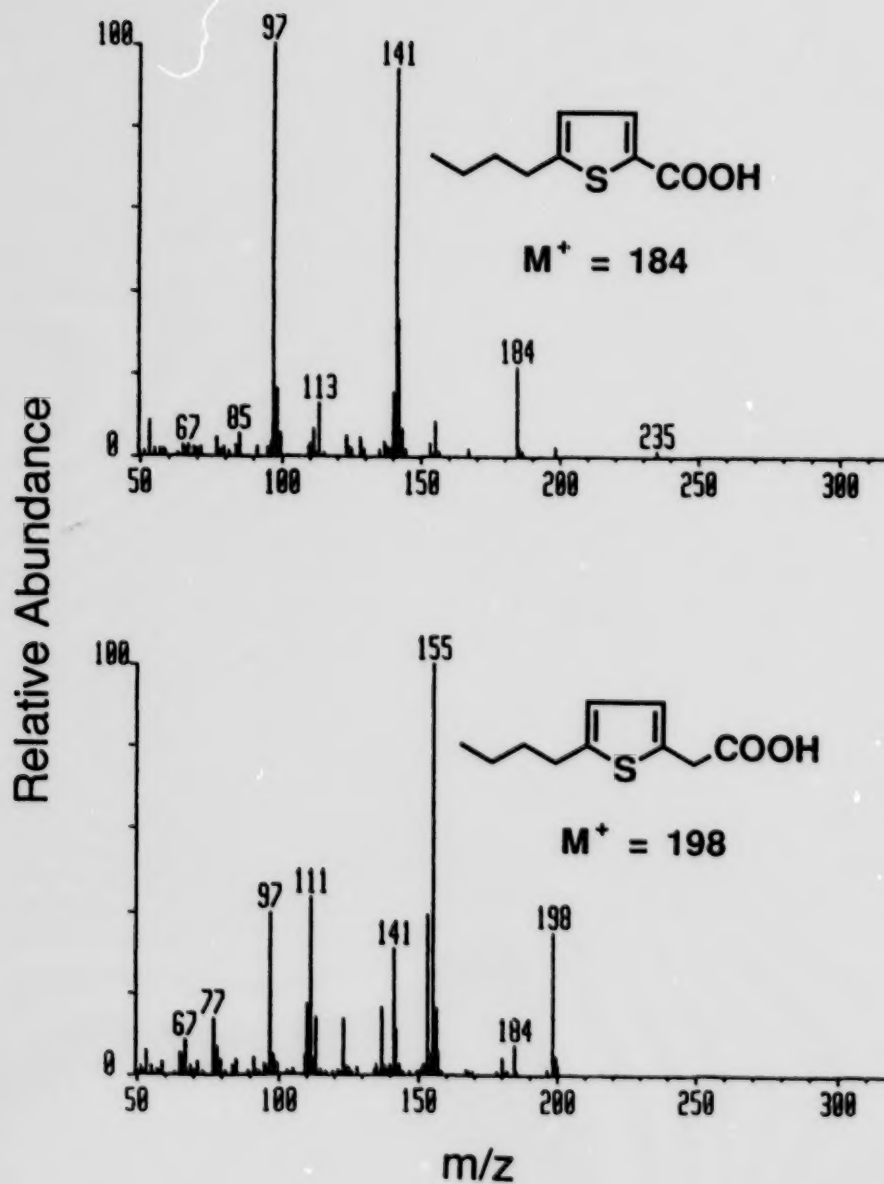


Figure 3.5. Mass spectra of the metabolites of compound T4-13 produced by isolate SE.

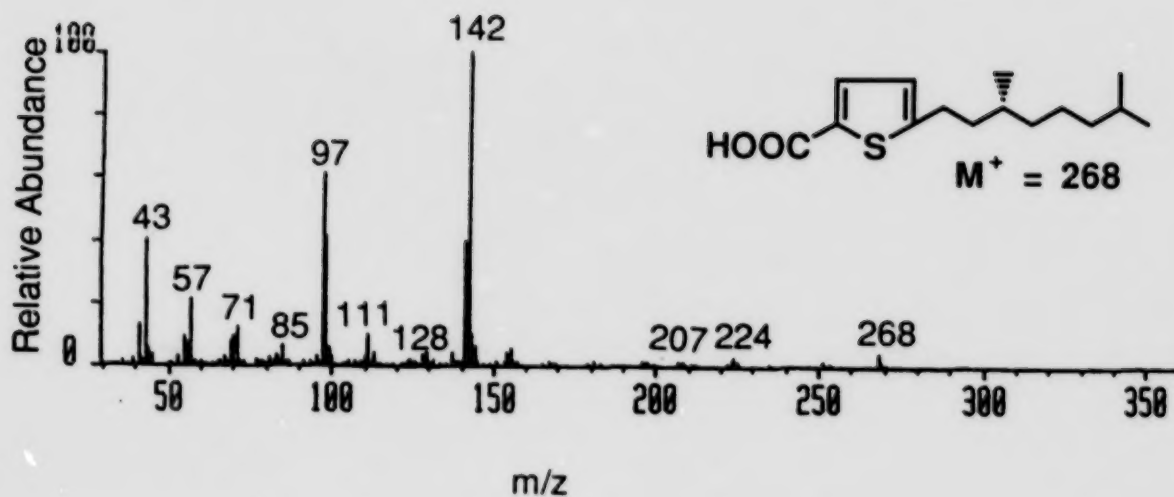


Figure 3.6. Mass spectrum and proposed structure of the metabolite of compound TMP29 produced by isolate SE grown on *n*-tetradecane in the presence of compound TMP29.

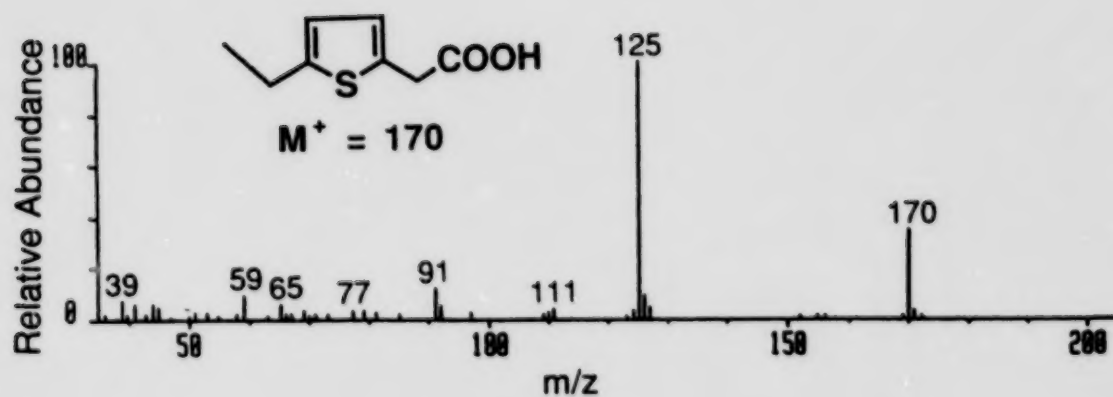


Figure 3.7. Mass spectrum and proposed structure of the metabolite of compound TMP40 produced by isolate SE grown on *n*-tetradecane in the presence of compound TMP40.

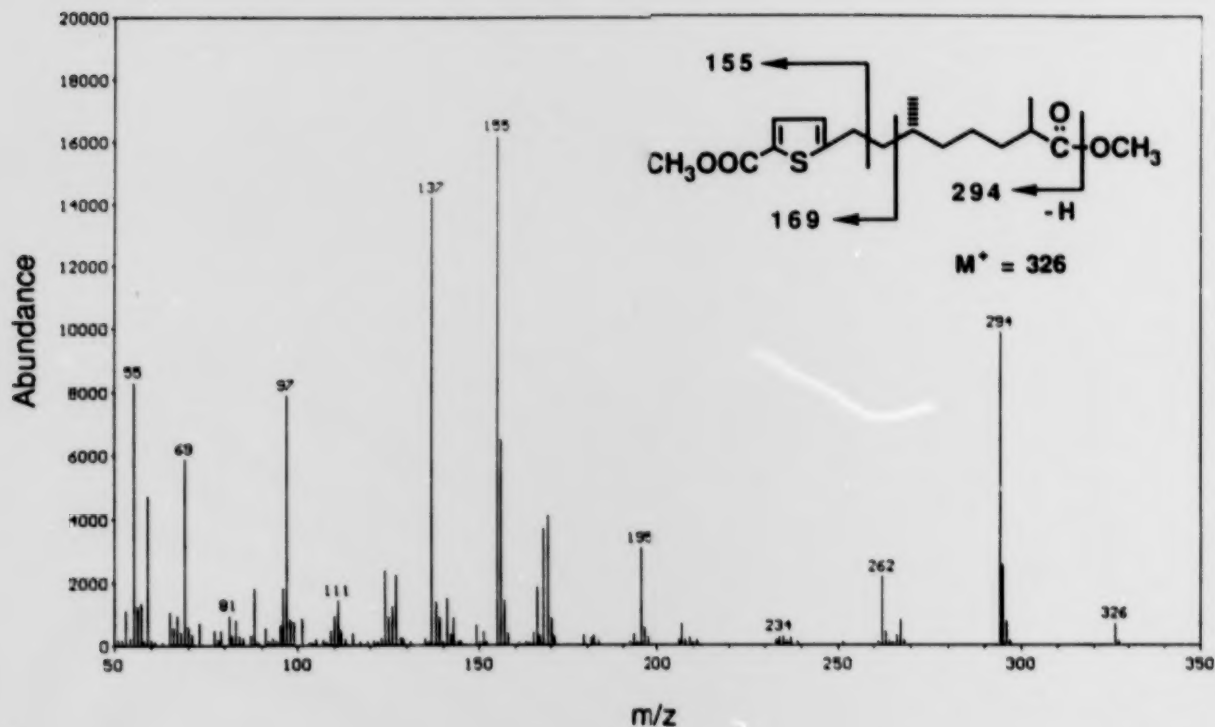


Figure 3.8. Mass spectrum and proposed structure of the methyl ester of the metabolite from compound TMP29 produced by isolate C2 grown on Prudhoe Bay crude oil in the presence of compound TMP29.

4.0 RESULTS FROM STUDIES WITH ALKYL BENZOTHIOPHENES

The studies on the biotransformations of the alkyl benzothiophenes used a bacterial isolate designated BT1. This *Pseudomonas* sp. was obtained from enrichment cultures inoculated with fuel-contaminated beach material from Shell Lake, Northwest Territories, Canada. Isolate BT1 has been shown to degrade a variety of aromatic hydrocarbons (Fedorak and Grbic'-Galic' 1991) and experiments with Norman Wells oil containing ^{14}C -labeled compounds showed that BT1 was able to mineralize aromatic hydrocarbons but not saturated hydrocarbons.

Fedorak and Grbic'-Galic' (1991) showed that BT1 could not grow on benzothiophene (BT) or 3-methylbenzothiophene (3-MBT). However, when grown on 1-methylnaphthalene (1-MN), these two organosulfur compounds were cometabolized and the products are shown in Figure 4.1. On the basis of this previous work, it was predicted that those alkyl benzothiophenes with methyl groups on the thiophene ring would yield the corresponding sulfoxides and sulfones, whereas those with the methyl groups on the benzene ring would yield 2,3-diones.

Isolate BT1 was inoculated into 500-mL screw-cap flasks containing 200 mL of liquid mineral medium (Fedorak *et al.* 1988) supplemented with trace metals solution (Fedorak and Grbic'-Galic' 1991), 50 μL of 1-MN and a few micrograms of an alkyl benzothiophene. The cultures were incubated for 14 days at 28°C with shaking. 1-MN was chosen as a substrate because it is a liquid at room temperature and it readily dissolves the organosulfur compounds making them available for microbial metabolism. In addition, the identities of the intermediates of 1-MN metabolism are known (Dean-Raymond and Bartha 1975) and they can easily be distinguished from metabolites of the benzothiophenes. Early studies used screw-cap flasks to prevent the evaporative loss of the alkyl benzothiophenes. The caps were loosened every day or two to replenish the headspace with oxygen. Later, foam plugs were used in place of the screw caps, although some evaporative loss of organosulfur compounds was observed.

Initially this part of the project was hampered by the repeated appearance of 3-MBT in the extracts of cultures that had not received this compound. Much time was spent trying to locate the source of the contaminant which hindered our progress for a few months. Initially we hypothesized that the 3-MBT adsorbed to the liners in the screw caps and diffused into the culture medium when we reused the caps for further experiments. Thorough washing of the screw caps used on the culture flasks and rigorous rinsing with solvents did not eliminate the contamination problem. Subsequently we used new Teflon-lined screw caps and discarded them after each experiment. However, the sulfur-containing contaminant persisted. We purchased a new batch of 1-MN and tests with cultures grown on this batch showed no 3-MBT contaminant whereas those grown on the old batch of 1-MN had the contaminant. Thus it appeared that the 1-MN had been contaminated with 3-MBT.

4.1 Decomposition of 3-methylbenzothiophene sulfoxide and 2-methylbenzothiophene sulfoxide in the GC injection port.

Andersson (1984) and Vignier *et al.* (1983) have reported that dibenzothiophene sulfoxide decomposes in the injection port of a GC and that the extent of decomposition depends on the type of injection port liner being used. The decomposition of dibenzothiophene sulfoxide yields dibenzothiophene and dibenzothiophene sulfone.

As our investigations progressed, there was evidence that 3-MBT sulfoxide decomposed in the same manner. Figure 4.2 shows a FPD chromatogram of the extract of a culture grown on 1-MN in the presence of 3-MBT. Typically the parent compound 3-MBT was observed in these chromatograms and it was assumed that the bacterial cometabolism left some of this compound unoxidized. When silica gel column chromatography was used to separate the polar sulfoxide and sulfone from the 3-MBT, subsequent GC analysis of the polar fraction gave a chromatogram that was essentially the same as Figure 4.2 showing a peak corresponding to 3-MBT.

Andersson (1984) demonstrated that the type of GC injection port used affected the amount of decomposition of dibenzothiophene sulfoxide. We analyzed the polar fraction using the standard Hewlett-Packard Jennings/cup mixing chamber (part no. 18740-80190) injection port liner under three different conditions. Based on the FID response we calculated the peak area ratios of 3-MBT to 3-MBT sulfoxide and of 3-MBT sulfone to 3-MBT sulfoxide. Duplicate injections were done under each condition. Considering the 3-MBT to 3-MBT sulfoxide ratios, when a packed liner which had been used for dozens of injections of petroleum samples and fractions was tested, the mean ratio was 16.6. When a clean liner without packing was tested, the mean ratio was 0.10. Finally, when the clean liner freshly packed with 10% OV-1 on 80/100 mesh Chromosorb-WHP as suggested by Hewlett-Packard was tested, the mean ratio was 0.18. The mean sulfone to sulfoxide ratios under the three conditions were 2.85, 0.25 and 0.27, respectively. Clearly the amounts of 3-MBT and its sulfone observed were dependant on the condition of the injection port liner.

The polar fraction obtained from the silica gel chromatography was also analyzed by HPLC and the resulting chromatogram is shown in Figure 4.3a. Decomposition of the sulfoxide and the sulfone would not occur during HPLC analysis. The sulfoxide had a retention time of 3.52 min and the sulfone had a retention time of 4.65 min. Authentic 3-MBT eluted at 28.18 min under these chromatographic conditions. 3-MBT was not detected in this polar fraction.

Fractions of the effluent from the HPLC column corresponding to 3-MBT sulfoxide and 3-MBT sulfone were collected and analyzed by GC. The fraction that contained only the 3-MBT sulfoxide gave a GC chromatogram similar to Figure 4.2, in which the sulfone and 3-MBT were formed by decomposition in the injection port. GC analysis of the HPLC fraction containing the 3-MBT sulfone

showed only one peak which corresponded to the sulfone.

In addition, the polar fraction of the methylene chloride extract from cultures of BT1 grown on glucose in the presence of 2-MBT was isolated and analyzed by HPLC and GC in the same manner as was done with the metabolites from 3-MBT. Under the HPLC conditions used, authentic 3-MBT had a retention time of 12.70 min. Figure 4.3b shows the HPLC chromatogram of the polar fraction and no 2-MBT was detected. However, 2-MBT was observed in the GC analysis of this fraction thereby demonstrating that 2-MBT sulfoxide is susceptible to decomposition in the GC injection port.

From these studies it was evident that the appearance of sulfones in the GC analysis of extracts from cultures grown in the presence of other methylbenzothiophenes may be due to the decomposition of the corresponding sulfoxide in the injection port. Thus when a sulfone was detected by GC analysis, its presence in the culture extract was verified by HPLC analysis.

4.2 Cometabolism of 2-methylbenzothiophene (2-MBT)

GC analysis of methylene chloride extracts of isolate BT1 grown on 1-MN with 2-MBT showed two sulfur-containing compounds which were poorly resolved. The more abundant metabolite eluted first at 21.9 min and had a peak area of about 10 times that of the other metabolite that eluted on its tail. The mass spectrum of the more abundant metabolite is shown in Figure 4.4b. It had a weak molecular ion of m/z 164 suggesting that the metabolite was a sulfoxide. Chemical ionization GC-MS, using isobutane as the reagent gas, verified that the molecule weight of this compound was 164.

Figure 4.4a shows the mass spectrum of 3-MBT sulfoxide that had been identified in previous studies (Fedorak and Grbic-Galic 1991). The base peaks in both of these mass spectra (Figures 4.4a and 4.4b) were m/z 147 which corresponds to the loss of -OH. Porter (1967) observed the predominant ion of m/z 147 in the mass spectra of 2-MBT and 3-MBT and considered these to be the benzothiopyrilium cation. The ion at m/z 121 observed in Figures 4.4a and 4.4b and in Porter's study was formulated to be C_7H_5S (Porter 1967). Although the mass spectra of 2-MBT and 3-MBT were virtually identical (Porter 1967) there were marked differences between those of the sulfoxides shown in Figures 4.4a and 4.4b.

The extract of isolate BT1 grown of 1-MN with 2-MBT was also analyzed by GC-FTIR. Figure 4.5a is the FTIR spectrum of the more abundant metabolite. It shows a single strong absorption at 1080 cm^{-1} , which is characteristic of a sulfoxide (Shriner *et al.* 1980). The FTIR results provide further evidence that the more abundant metabolite was 2-MBT sulfoxide.

The syntheses of the sulfoxides of benzothiophene or alkyl benzothiophenes are difficult because most available oxidation procedures yield the corresponding sulfones (ie. the dioxides) (Andersson personal communication; Wey personal communication). Therefore, no authentic

standards were available for comparison with these metabolites.

GC-MS analysis gave no evidence of the presence of 2-MBT sulfone, presumably because the column used was not giving sufficient resolution of the sulfoxide and the sulfone. However, GC-FTIR analysis detected the presence of a minor peak and its FTIR spectrum is shown in Figure 4.5b. The spectrum of this metabolite of 2-MBT shows two strong absorptions at 1162 and 1336 cm^{-1} which are characteristic of a sulfone (Shriner *et al.* 1980).

4.3 Cometabolism of a mixture of 4- and 6-methylbenzothiophene (4-MBT and 6-MBT)

The synthesis procedure used by Andersson (1986) yielded a mixture of these two isomers and therefore the mixture was added to a culture of BT1 growing on 1-MN. Figure 4.6 shows the FPD chromatogram of the methylene chloride extract of this culture after 14 days incubation. Three sulfur-containing metabolites were observed and these were designated Compounds 1, 2 and 3.

The mass spectrum of Compound 1 (Figure 4.7a) shows a molecular ion of m/z 154 corresponding to an empirical formula of $\text{C}_8\text{H}_{10}\text{OS}$. The predominant ion observed from chemical ionization GC-MS using isobutane as the reagent gas was $(M+1)^+$ 155, confirming that the molecular weight of the metabolite was 154. The FTIR spectrum of this metabolite (Figure 4.7b) shows a strong absorption at 1099 cm^{-1} which is characteristic of a sulfoxide, an ether or a secondary alcohol although there is no strong O-H stretch at 3200-3600 cm^{-1} .

A TLC method was developed for the isolation of analytical amounts of Compound 1. Commercially available silica gel plates containing a fluorescent indicator (BAKER, Si250F glass plates, Canlab) were activated at 120°C for 4 h prior to use. After applying a few microlitres of a culture extract, the TLC plate was developed with 1% methanol in ethylacetate. The plate was observed under visible light, long-wave and short-wave UV light. A total of eleven spots were seen on the plate. One spot, which was very well separated from the others, had an R_f of 0.53 and appeared pale gray under short-wave UV. After eluting the spot from the silica gel with methylene chloride, and concentrating the solution, GC analysis showed that the recovered material was Compound 1 based on retention time and response of the FPD.

A culture extract containing Compound 1 was oxidized using the method of Bordwell *et al.* (1949) to give the corresponding sulfone. Electron impact GC-MS showed the molecular weight of the sulfone of the metabolite was 170. This was confirmed by chemical ionization GC-MS using ammonia as the reagent which gave a $(M+18)^+$ ion at m/z 188. Thus the molecular weight of the sulfone obtained from oxidation of Compound 1 was 16 mass units higher than that of Compound 1. This indicated that Compound 1 was not a sulfide-containing alcohol because the oxidation of such an alcohol would yield a product with molecular weight of 32 mass units greater than the reactant.

These results suggested that Compound 1 was *m*-tolyl methyl sulfoxide which could arise from either 4-MBT or 6-MBT. High resolution mass spectrometry indicated that the base peak at m/z 139 (Figure 4.7) was C_7H_7OS . The ions at m/z 111 and 108 corresponded to the fragments C_6H_7S , and C_7H_8O , respectively. The ions at m/z 91, 77 and 65 are characteristic of aralkyl hydrocarbons (Silverstein and Bassler 1967) and correspond to the fragments C_7H_7 (the tropylium ion), C_6H_5 and C_5H_5 , respectively. The fragmentation pattern of the mass spectrum of this metabolite (Figure 4.7) matched that of phenyl methyl sulfoxide given by Bowie *et al.* (1966). For example, the parent ion, $(M-15)^+$, at m/z 139 resulted from the loss of the methyl group. Bowie *et al.* (1966) show that the $(M-15)^+$ ion was predominant in the mass spectrum of phenyl methyl sulfoxide. The subsequent loss of $-CO$ gives the $(M-43)^+$ ion at m/z 111 observed in Figure 4.7 and at m/z 97 observed for phenyl methyl sulfoxide (Bowie *et al.* 1966).

Subsequently, *m*-tolyl methyl sulfoxide and *m*-tolyl methyl sulfone were synthesized. Indeed, Compound 1 and its oxidation product had the same GC retention times and mass spectra as the authentic sulfoxide and sulfone, respectively. The appearance *m*-tolyl methyl sulfoxide in cultures grown in the presence of 4- and 6-MBT suggests that the benzothiophene ring was broken. However, the absence of any C-O bonds in the ring-cleavage product is unusual, and the mechanism by which this compound was formed is unknown.

Compounds 2 and 3 have virtually the same mass spectra (Figures 4.8b and 4.8c) and very similar FTIR spectra (Figure 4.9b and 4.9c). The mass spectra of Compounds 2 and 3 show a weak molecular ion of m/z 178 suggesting that these two metabolites were the diones of 4-MBT and 6-MBT. These have a similar fragmentation patterns to that of authentic benzothiophene-2,3-dione (Figure 4.8a) which has a weak molecular ion of m/z 164 (14 mass units less than the metabolites of 4-MBT and 6-MBT). The base peaks of these metabolites were m/z 150 which corresponds to a loss of CO as was observed for benzothiophene-2,3-dione (m/z 136). A subsequent loss of CHO would yield the second most abundant ion at m/z 121 $(M-57)^+$. This fragmentation differs slightly from that of benzothiophene-2,3-dione in which there was a subsequent loss of CO rather than CHO resulting in the $(M-56)^+$ ion.

The FTIR spectra of Compounds 2 and 3 from 4- and 6-MBT were very similar to that of the authentic benzothiophene-2,3-dione as shown in Figure 4.9. The authentic 2,3-dione shows a strong absorption at 1736 cm^{-1} with a shoulder at about 1770 cm^{-1} (Figure 4.9a). Compound 2 shows a strong absorption at 1728 cm^{-1} with a shoulder at about 1760 cm^{-1} (Figure 4.9b) and Compound 3 shows a strong absorption at 1738 cm^{-1} with no shoulder (Figure 4.9c).

The GC-MS and GC-FTIR results indicate that Compounds 2 and 3 are isomers of methylbenzothiophene-2,3-dione. However, because the mixture of 4- and 6-MBT was used in this

culture, we could not determine which metabolite was 4-methylbenzothiophene-2,3-dione, and which was 6-methylbenzothiophene-2,3-dione.

4.4 Cometabolism of 4-methylbenzothiophene (4-MBT)

Because there were three sulfur-containing metabolites produced from the cometabolism of a mixture of 4-MBT and 6-MBT, a pure preparation of 4-MBT was used to help elucidate the source of Compound 1. When the methylene chloride-extract from a culture grown on 1-MN in the presence of pure 4-MBT was analyzed by GC, the FPD showed only one metabolite. It had the same retention time as Compound 2, and subsequent GC-MS analysis showed it have the same mass spectrum as Compound 2. Thus Compound 2 was 4-methylbenzothiophene-2,3-dione and Compound 3 was 6-methylbenzothiophene-2,3-dione. In addition, these results indicate that Compound 1 was a metabolite of 6-MBT.

4.5 Cometabolism of 5-methylbenzothiophene (5-MBT)

Figures 4.10a and 4.10c show the mass spectrum and the FTIR spectrum, respectively, of the sulfur-containing metabolite found in the extract of the culture grown in the presence of 5-MBT. These results suggest that the metabolite was 5-methylbenzothiophene-2,3-dione.

5-Methylbenzothiophene-2,3-dione was synthesized (m.p. 143-146°C, lit. m.p. 143-144°C) (Hannoun *et al.* 1982) to unequivocally prove that the metabolite of 5-MBT was its 2,3-dione. The GC retention time of the metabolite was the same as that of the authentic 5-methylbenzothiophene-2,3-dione. In addition, the mass spectrum of the metabolite (Figure 4.10a) is virtually identical to that of the authentic standard (Figure 4.10b). There was a weak molecular ion of m/z 178; the parent peak of m/z 150 resulting from the loss of CO; and another abundant ion of m/z 121 resulting from the subsequent loss of CHO.

The FTIR spectrum of the metabolite of 5-MBT shows a strong absorption at 1737 cm^{-1} (Figure 4.10c) which is virtually identical to that of authentic 5-methylbenzothiophene-2,3-dione (Figure 4.10d). The results from GC-MS and GC-FTIR analyses clearly show that the metabolite produced from 5-MBT is its 2,3-dione.

4.6 Cometabolism of 7-methylbenzothiophene (7-MBT)

Figure 4.11 shows the GC analysis of the sulfur-containing metabolites found in the extract of isolate BT1 grown on 1-MN in the presence of 7-MBT. This was the most complex chromatogram observed in this study. Twelve peaks have been labeled (A to L) and we attempted to identify some of these compounds. The tentative identification of these metabolites using GC-FPD, GC-MS and GC-FTIR was complicated by difficulties reproducing the chromatograms obtained with the three

different instruments, each with a slightly different GC column. Only a few of the twelve metabolites are considered in this report.

Because of the prevalence of diones, sulfoxides and sulfones observed with other methylbenzothiophenes, these were the first compounds for which we searched. Figure 4.12 shows the MS and FTIR spectra of Compound G. The FTIR spectrum of compound G shows two strong absorptions at 1334 and 1164 cm^{-1} and was very similar to that of authentic 7-MBT sulfone synthesized (Figure 4.13b). The mass spectrum of compound G (Figure 4.12a) shows a molecular ion (m/z 180) that is consistent with the metabolite being 7-MBT sulfone. However, the mass spectrum of Compound G differs from that of authentic 7-MBT sulfone (Figure 13a), presumably because of the presence of a contaminating compound eluted from the GC column along with Compound G.

Based on GC-MS and GC-FTIR analyses (Figure 4.14), Compound F appears to be the sulfoxide of 7-MBT. The mass spectrum of Compound F was similar to that of the sulfoxide of 2-MBT shown in Figure 4.4a and the FTIR shows a strong absorption at 1079 cm^{-1} which is consistent with the absorption of the sulfoxide of 2-MBT shown in Figure 4.5a.

The mass spectrum of Compound D (Figure 4.15a) shows a weak molecular ion at m/z 178, the parent peak at m/z 150 and a major peak at m/z 121. The similarities between this spectrum and that of 5-MBT-2,3-dione (Figure 4.10a) suggested that the metabolite was 7-MBT-2,3-dione. Therefore we synthesized 7-methylbenzothiophene-2,3-dione (m.p. 126°C, lit. m.p. 126-127°C) to unequivocally identify Compound D. The mass spectrum of the metabolite (Figure 4.15a) contains several fragment ions that were not evident in that of the authentic standard (Figure 4.15c) and these may be due to another compound from the culture extract that is co-eluting with Compound D. Nonetheless, the FTIR spectrum of Compound D (Figure 4.15b) is virtually identical to that of the authentic standard (Figure 4.15d). These results clearly show that Compound D is 7-MBT-2,3-dione.

The GC and FTIR spectra of two other metabolites from 7-MBT are given in Figures 4.16 and 4.17. The molecular ion of Compound B is likely m/z 160 and the parent peak is at m/z 118 ($M-42$)⁺ (Figure 4.16a). The FTIR shows a strong absorption at 1748 cm^{-1} with relatively few other absorptions. The identity of Compound B is unknown.

Figure 4.17 shows the MS and FTIR spectra of Compound E which has a strong molecular ion at m/z 180 suggesting that cometabolism lead to the addition of two atoms of oxygen to 7-MBT giving the formula $\text{C}_9\text{H}_8\text{O}_2\text{S}$. The parent peak is at m/z 147 ($M-33$)⁺ indicating that this metabolite has a different fragmentation pattern than 3-MBT sulfone (Fedorak and Grbic'-Galic' 1991) or 2,3-diones of 4-, 6- and 5-MBT (Figures 4.9b, 4.9c and 4.10b, respectively). The FTIR spectrum of compound E (Figure 4.17b) is distinctly different from any of the others spectra of metabolites that have been observed. The strong absorption at 1749 cm^{-1} suggests the presence of a ketone and the

absorptions at 1151 and 2978 cm^{-1} suggest the presence of a hydroxyl group.

Further work is required to identify many of the sulfur-containing compounds observed in the extract from cultures grown with 7-MBT in the medium. However, the presence of a dione, a sulfoxide and a sulfone in the culture extract was unique among the methylbenzothiophenes observed to date.

4.7 Cometabolism of 2,3-dimethylbenzothiophene (2,3-DMBT)

GC analysis of methylene chloride extracts of isolate BT1 grown on 1-MN with 2,3-DMBT showed two sulfur-containing metabolites. The major metabolite eluted at a retention time of 26.2 min while the minor metabolite eluted at a retention time of 26.7 min. GC-MS analysis showed that the major metabolite had a weak molecular ion of $m/z = 178$ (Figure 4.18a) suggesting that the metabolite was the sulfoxide of 2,3-DMBT. The fragment at m/z 163 corresponds to the loss of $-\text{CH}_3$ and the fragment at m/z 145 suggests a subsequent loss of H_2O . When this extract was analyzed by GC-FTIR, the spectrum of the major metabolite showed a single strong absorption at 1070 cm^{-1} , which is characteristic of sulfoxide. These results suggest that the major metabolite of 2,3-DMBT was its sulfoxide.

The mass spectrum of the minor metabolite showed a molecular ion of m/z 194 and a base peak of m/z 151. This spectrum was virtually identical to the mass spectrum of an authentic standard of 2,3-DMBT sulfone which was provided by Dr. Andersson. In addition, the GC retention times of the minor metabolite and the authentic standard were the same. The FTIR spectrum of the minor metabolite showed two strong absorptions at 1170 and 1321 cm^{-1} as would be expected for a sulfone. Thus the minor metabolite was 2,3-DMBT sulfone.

4.8 Cometabolism of 2,7-dimethylbenzothiophene (2,7-DMBT)

GC analysis of the methylene chloride extract of isolate BT1 grown for 14 days on 1-MN with 2,7-DMBT showed a major sulfur-containing metabolite. The mass spectrum of this compound is shown in Figure 4.18b. The molecular ion appeared at m/z 178 and this was consistent with the metabolite being the sulfoxide of 2,7-DMBT. The fragments at m/z 163 and m/z 161 correspond to the losses of $-\text{CH}_3$ and $-\text{OH}$, respectively. The base peak at m/z 135 may be the result of the loss of a fragment containing the oxygen atom, the C-2 carbon atom and the C-2 methyl group $(\text{M}-43)^+$. Thus the parent ion would be 14 mass units larger than the m/z 121 ion observed for 2-MBT sulfoxide in Figure 4.4b and by Porter (1967) for 2-MBT. In addition, the m/z 43 ion, corresponding to $\text{C}_2\text{H}_3\text{O}$, was very abundant.

GC-FTIR analysis of this extract yielded the spectra shown in Figure 4.19. Careful scrutiny of

the predominant GC peak showed that different FTIR spectra existed within the peak indicating that two compounds were eluting together. Figure 4.19a shows the spectrum observed on the front portion of the peak. The predominant absorption at 1089 cm^{-1} was at essentially the same position as that observed for 2-MBT sulfoxide (Figures 4.5) indicating that the major metabolite was 2,7-DMBT sulfoxide. Figure 4.19b shows the spectrum obtained at the tail of the GC peak after appropriate background subtraction. The strong absorptions at 1327 and 1160 cm^{-1} are consistent with those found for 3-MBT sulfone (1339 and 1192 cm^{-1} ; Fedorak and Grbic-Galic 1991) and 2-MBT sulfone (1336 and 1162 cm^{-1} ; Figure 4.5b). There was no evidence of the sulfone from GC-MS analysis.

4.9 Cometabolism of 3,5-dimethylbenzothiophene (3,5-DMBT)

Figure 4.20 shows the sulfur-containing compounds found in the extract from a culture of isolate BT1 growing on 1-MN in the presence of 3,5-DMBT. The mass spectra and FTIR spectra of the three sulfur-containing compounds are shown in Figures 4.21-4.23. Of these three compounds, only the one designated A1 was identified and this metabolite was 5-MBT-2,3-dione. Peak A1 had the same GC retention time as authentic 5-MBT-2,3-dione. Also, the mass spectrum of compound A1 (Figure 4.21a) was virtually identical to that of 5-MBT-2,3-dione (Figure 4.10b). The FTIR spectrum of compound A1 (Figure 4.21b) has a strong adsorption at 1737 cm^{-1} and is very similar to the spectrum of 5-MBT-2,3-dione (Figure 4.10d) and almost identical to the metabolite obtained from 5-MBT (Figure 4.10c).

All other experiments with alkyl benzothiophenes containing methyl groups on the thiophene ring (2-MBT, 3-MBT, 2,3-DMBT and 2,7-DMBT) yielded sulfoxides and sulfones as cometabolism products of isolate BT1. Using the computer software available from the instrument manufacturer, the spectra obtained by GC-FTIR were scanned to detect any sulfoxides or sulfones produced from 3,5-DMBT. The selective wavenumber range for sulfoxides was $1050\text{-}1000\text{ cm}^{-1}$ and the ranges for sulfones were $1130\text{-}1200$ and $1300\text{-}1360\text{ cm}^{-1}$. This procedure worked well for the previous work with 7-MBT, but the selective scanning procedure detected no sulfoxide or sulfone from the cometabolism of 3,5-DMBT.

Clearly the transformation of 3,5-DMBT was unique among the alkyl benzothiophenes studied to date. To yield 5-methylbenzothiophene-2,3-dione, the methyl group on C-3 must be removed. This is the first instance of that methyl group loss during the cometabolism of these alkyl benzothiophenes. The elucidation of the structures of Compounds A2 and A3 in Figure 4.20 may help explain the mechanism of biotransformation of 3,5-DMBT.

4.10 Cometabolism of 3,7-dimethylbenzothiophene (3,7-DMBT)

The observation that the C-3 methyl group was removed from 3,5-DMBT prompted work with 3,7-DMBT to determine if the C-3 methyl group was also removed from this isomer. In one trial, the FPD chromatogram of the extract from isolate BT1 grown on 1-MN in the presence of 3,7-DMBT showed one large metabolite peak. GC-MS analysis revealed that the metabolite had a molecular ion at m/z 178 which was consistent with the compound being 3,7-DMBT sulfoxide. GC-FTIR analysis confirmed this identification showing a strong absorption at 1069 cm^{-1} . GC-FTIR analysis also showed that this peak also contained a second compound that showed two strong absorptions at 1160 cm^{-1} and 1327 cm^{-1} . This is characteristic of a sulfone. In a second trial, a small peak was observed that had the same retention time and mass spectrum as authentic 7-MBT-2,3-dione.

Although the dione was detected, indicating that the removal of the C-3 methyl group occurred, it was a very minor product. This was quite different from the situation with 3,5-DMBT where 5-MBT-2,3-dione was the major metabolite (peak A1, Figure 4.20).

4.11 Cometabolism of 7-ethylbenzothiophene (7-EBT)

Isolate BT1 was grown on 1-MN in the presence of 7-EBT and the extract has been analyzed by GC with the FPD. The first seven peaks in this chromatogram (not shown) gave essentially the same pattern as peaks A-G observed in Figure 4.11 for 7-MBT. Thus it appears that the substitution of carbon-7 of benzothiophene leads to a variety of metabolites. There was not sufficient time during this project to determine the identities of these metabolites.

4.12 Glucose as a substrate for the cometabolism of alkyl benzothiophenes.

In earlier studies on the cometabolism of benzothiophene and 3-MBT, Fedorak and Grbic-Galic (1991) grew a 1-MN-degrading enrichment culture on glucose (250 mg/L) with either of these two sulfur heterocyclic compounds in the medium. The culture, which was predominantly strain BT1, produced the same sulfur-containing metabolites when grown on glucose as it did when it was grown on 1-MN. This procedure was repeated using strain BT1 and the following alkyl benzothiophenes: 2-MBT, 4- and 6-MBT, 5-MBT, 7-MBT and 7-EBT. In each case, the metabolites observed in the culture grown on glucose were the same as those observed when it was grown on 1-MN. In addition, *m*-tolyl methyl sulfoxide was also found in the cultures grown on glucose in the presence of the mixture of 4- and 6-MBT. Thus, the presence of an aromatic hydrocarbon was not required for strain BT1 to transform these organosulfur compounds.

4.13 Summary

The data in Table 4.1 summarizes the identities and numbers of sulfur-containing metabolites found in the cultures of BT1 grown on 1-MN in the presence of various alkyl benzothiophenes. With two exceptions, these results agree with the prediction that those alkyl benzothiophenes with methyl groups on the thiophene ring would yield sulfoxides and sulfones, whereas those with the methyl groups on the benzene ring would yield 2,3-diones. The exceptions were: (1) 7-MBT which yielded a variety of metabolites including the corresponding sulfoxide, sulfone and dione; and (2) 3,5-DMBT which yielded a dione rather than the predicted sulfoxide and sulfone. Preliminary data from 7-EBT indicates a similar range of metabolites may be found.

Table 4.1. Products of the cometabolism of various methylbenzothiophenes by strain BT1.

Substrate	2,3-Dione	Sulfoxide	Sulfone	Other S-containing Metabolites
3-MBT	-	+++	+ ^a	0
2-MBT	-	+++	+ ^a	0
2,3-DMBT	-	+++	+ ^a	0
2,7-DMBT	-	+++	+ ^a	0
3,7-DMBT	±	+++	+	0
3,5-DMBT	+++	-	-	2
Benzothiophene	+++	-	-	0
4-MBT & 6-MBT	+++ & +++	-	-	1 ^b
4-MBT	+++	-	-	0
5-MBT	+++	-	-	0
7-MBT	+	+++	+++ ^a	9

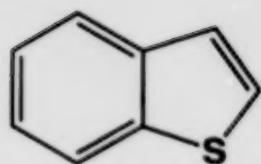
- = not detected

+ = small quantity detected

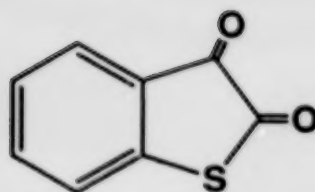
+++ = major metabolite detected

^a = verified by HPLC analysis.

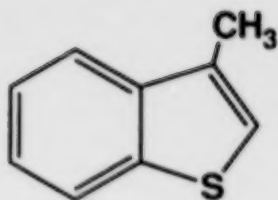
^b = other metabolite was identified as *m*-tolyl methyl sulfoxide.



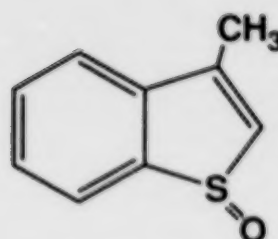
Benzothiophene



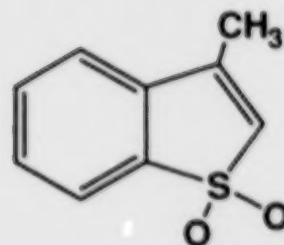
Benzothiophene-2,3-dione



3-Methylbenzothiophene



3-Methylbenzothiophene-sulfoxide



3-Methylbenzothiophene-sulfone

Figure 4.1. Cometabolism of benzothiophene and 3-methylbenzothiophene as reported by Fedorak and Grbic-Galic (1991).

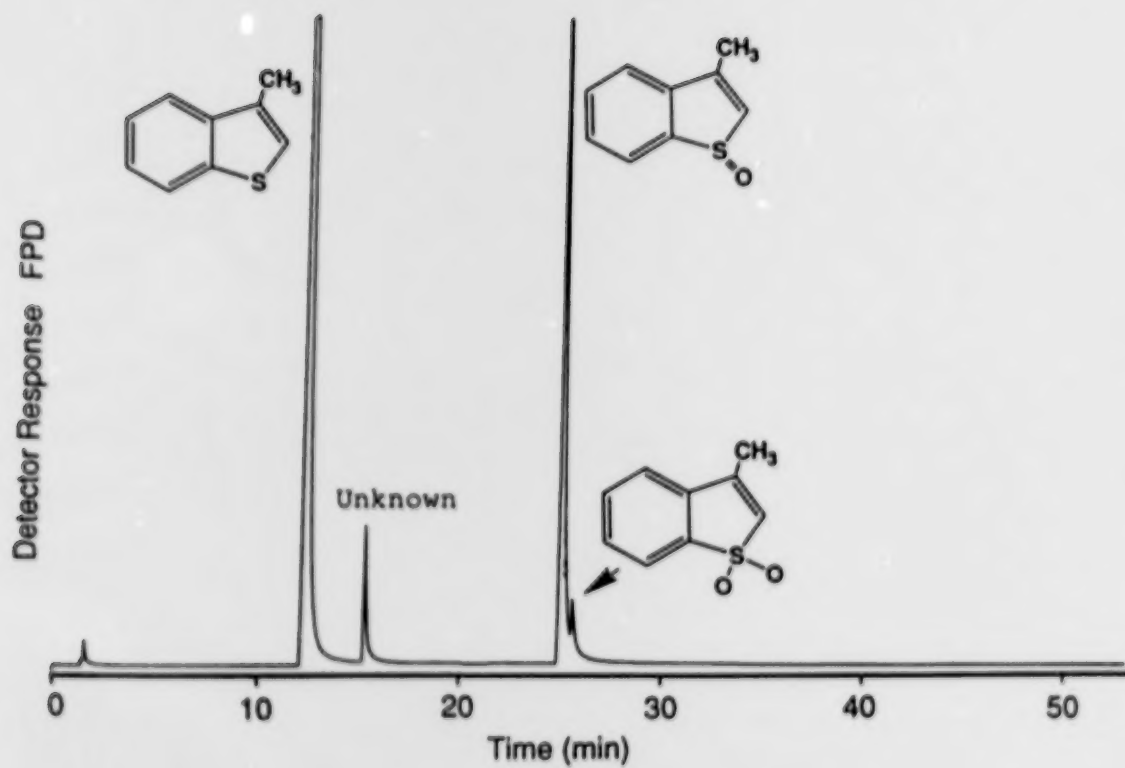


Figure 4.2. GC analysis of the methylene chloride extract of isolate BT1 grown on 1-MN in the presence of 3-MBT.

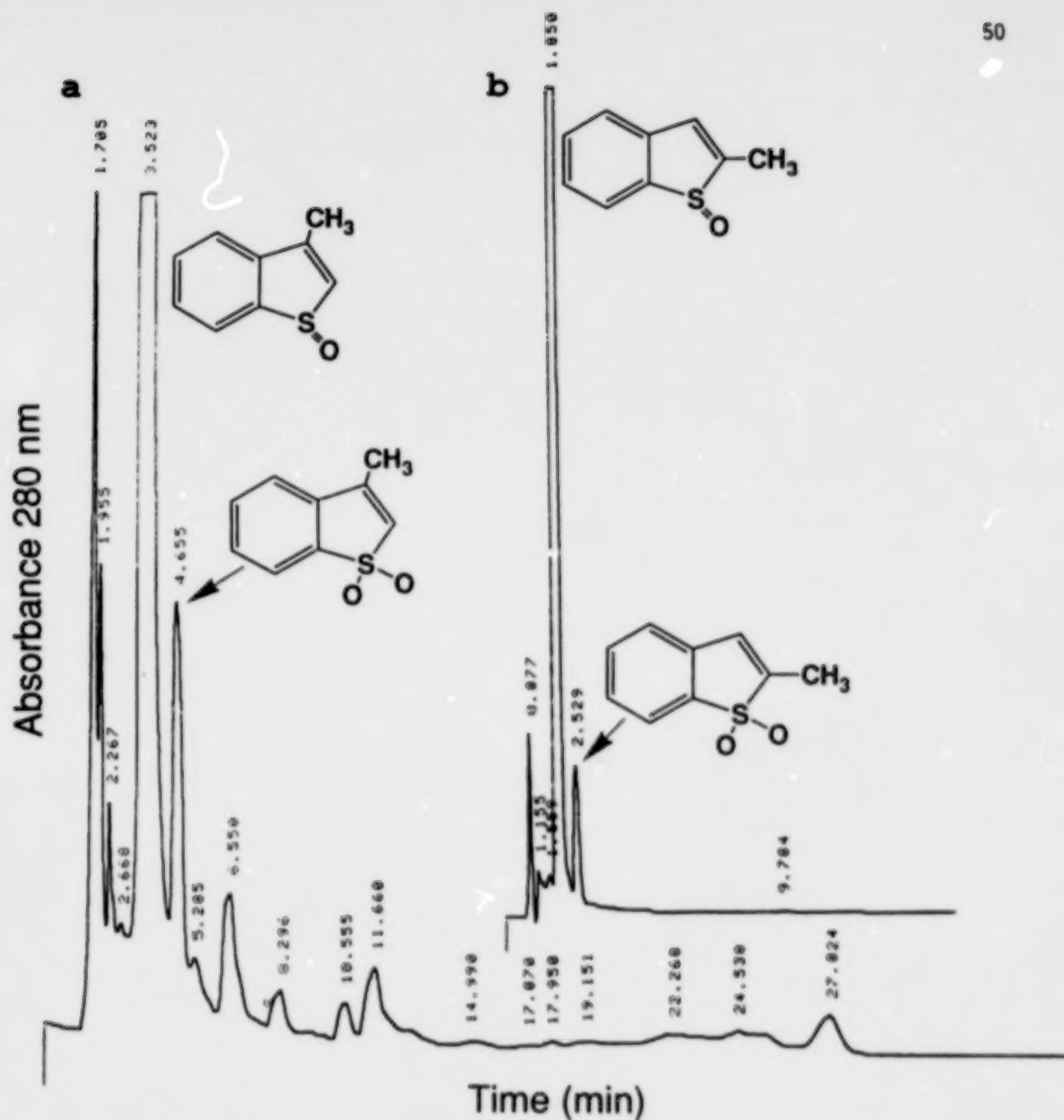


Figure 4.3. HPLC analyses of the polar fraction of extracts from cultures of isolate BT1 grown (a) on 1-MN in the presence of 3-MBT and (b) on glucose in the presence of 2-MBT. The polar fraction was obtained by silica gel column chromatography. Solvent flow rates were (a) 0.5 mL/min, (b) 1.0 mL/min.

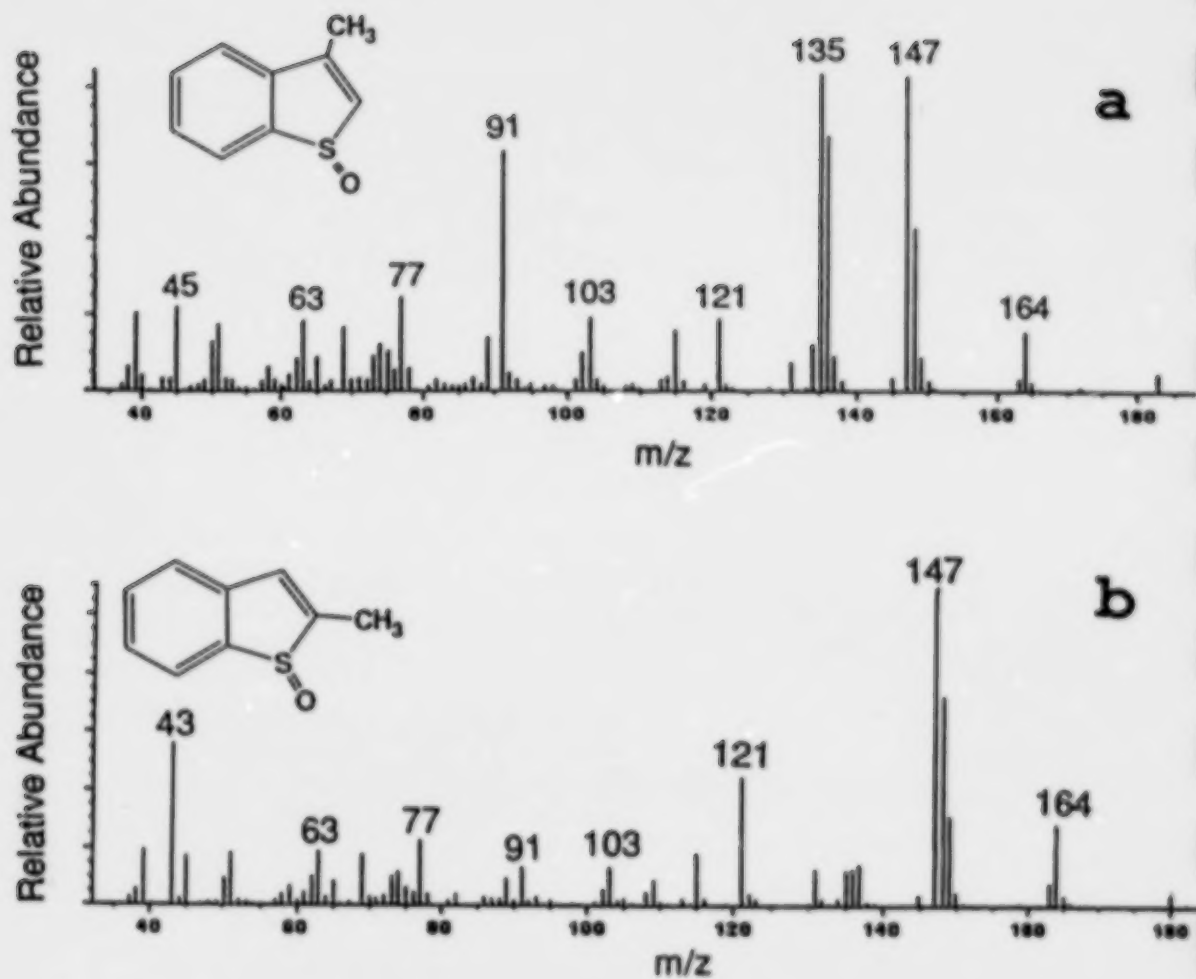


Figure 4.4. Mass spectra of sulfones of (a) 3-MBT and (b) 2-MBT that were produced as metabolites by isolate BT1 grown on 1-MN.

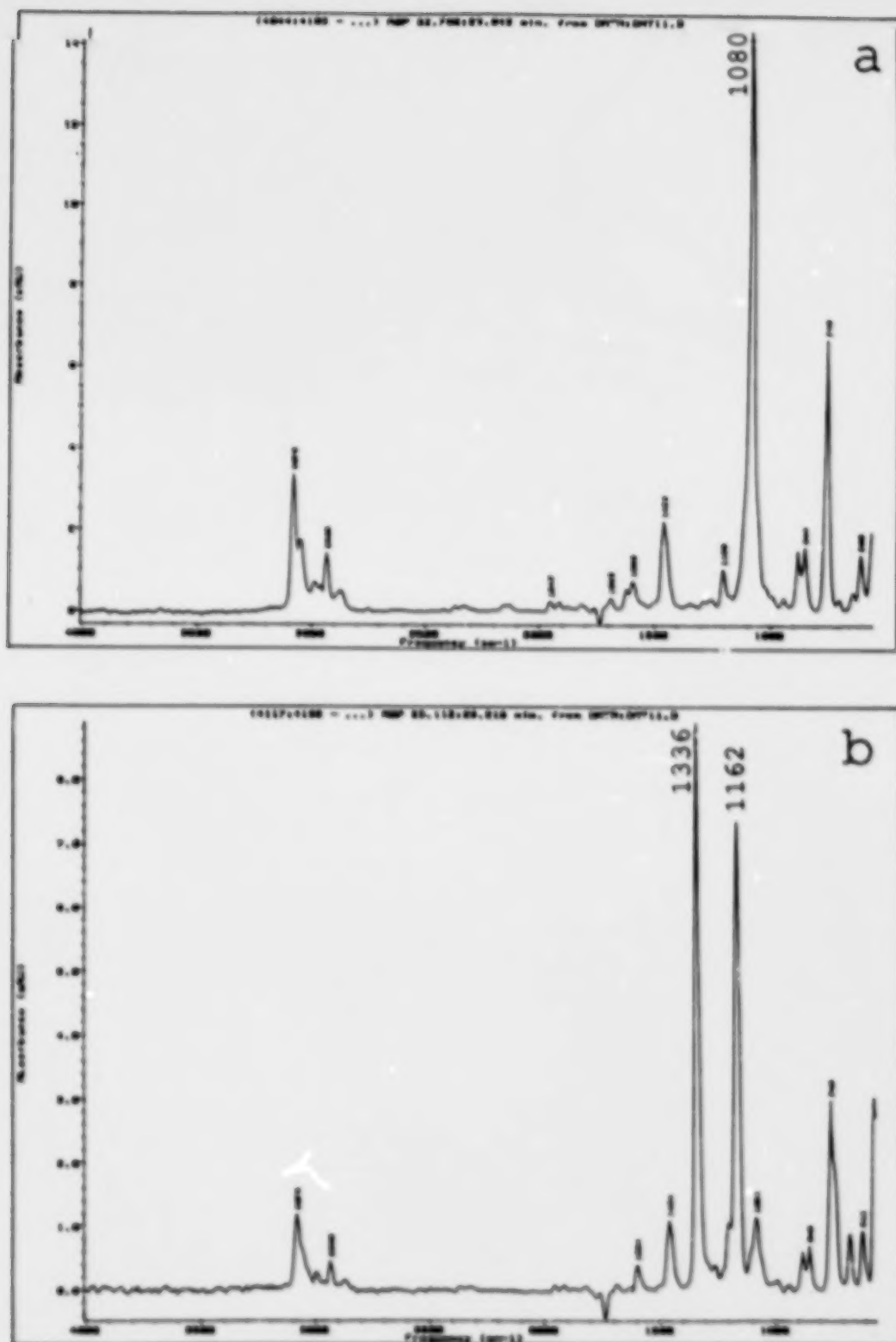


Figure 4.5. FTIR spectra of the metabolites of 2-MBT; (a) sulfoxide, (b) sulfone.

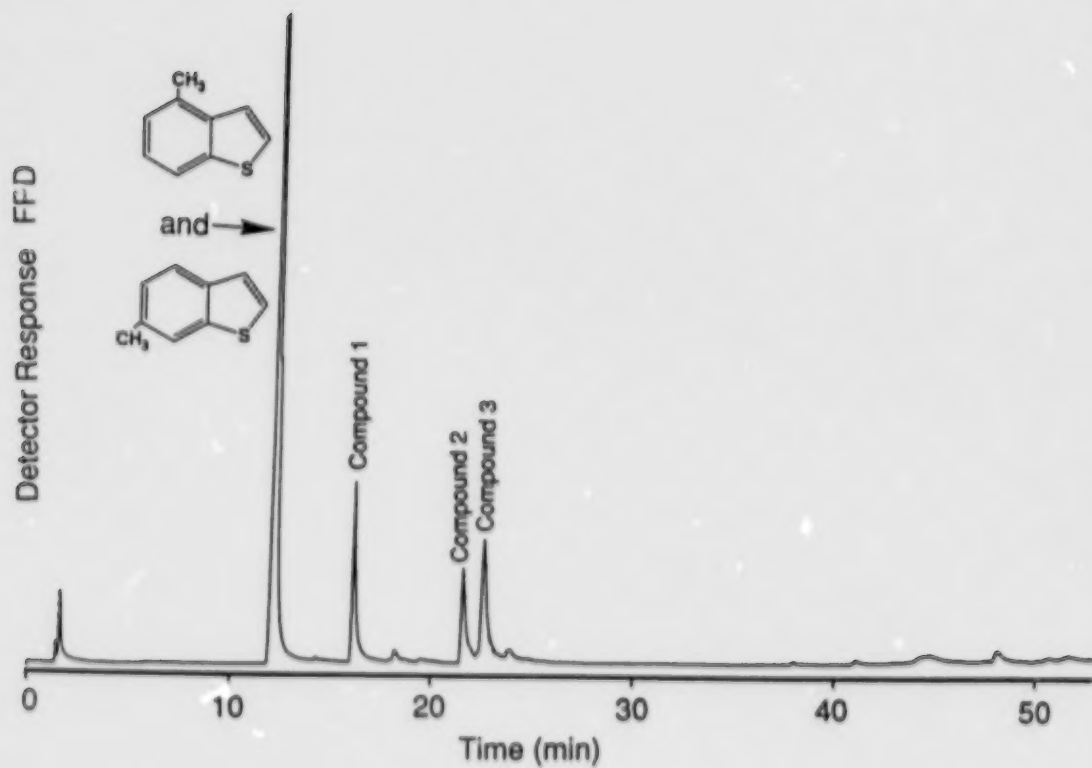


Figure 4.6. GC analysis of the methylene chloride extract of isolate BT1 grown on 1-MN in the presence of a mixture of 4- and 6-MBT.

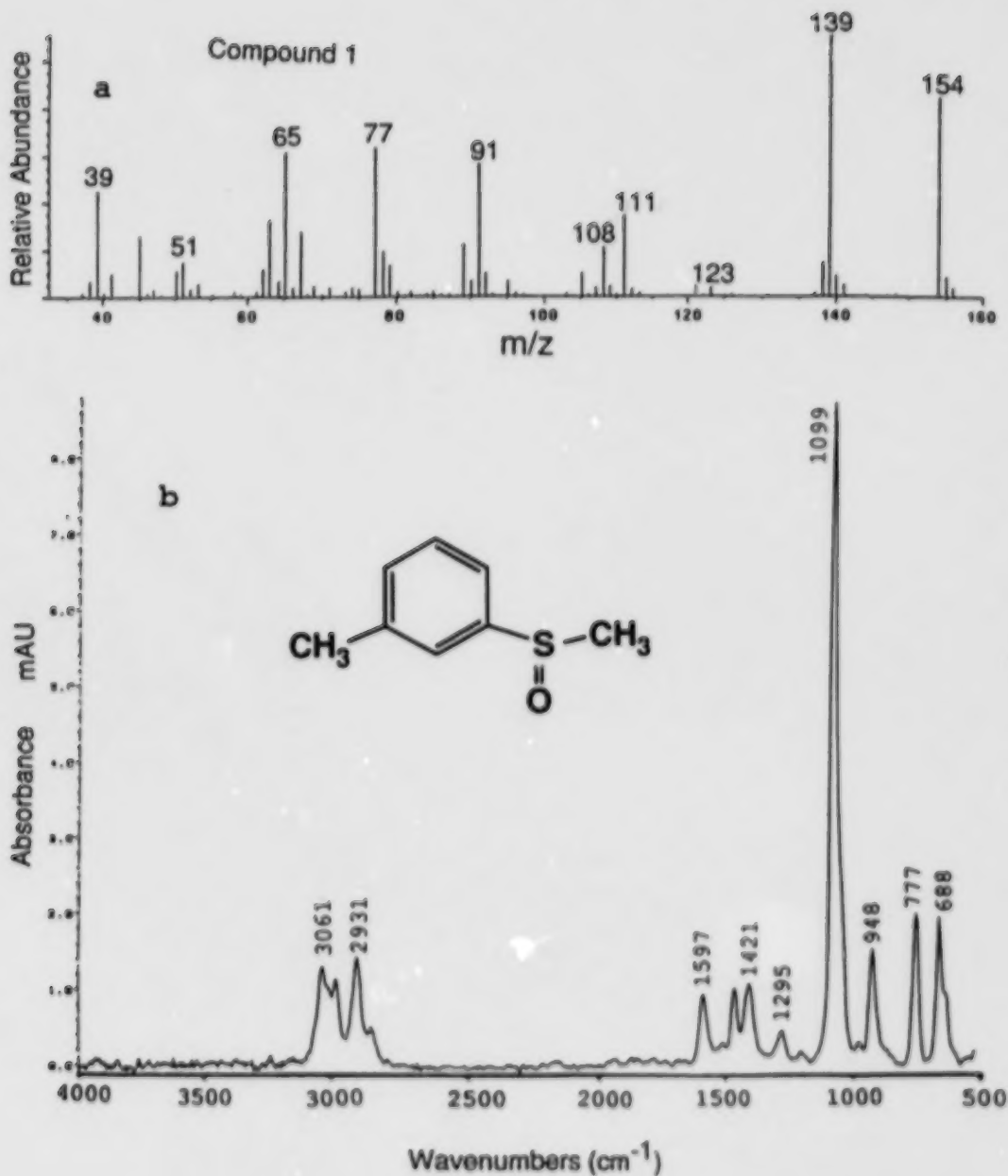


Figure 4.7. Spectra of Compound 1 obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 4- and 6-MBT; (a) mass spectrum, (b) FTIR spectrum.

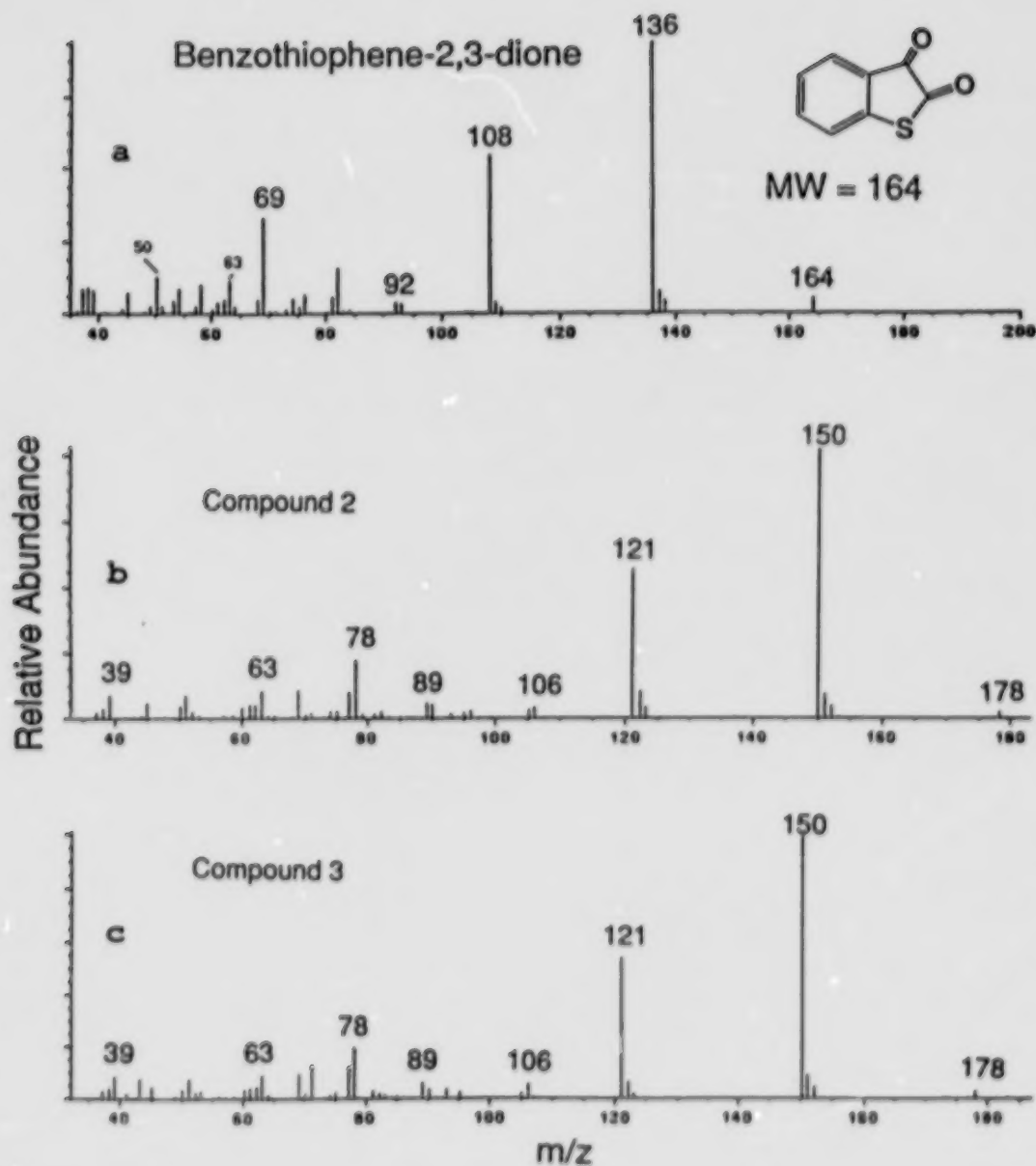


Figure 4.8. Mass spectra of (a) authentic benzothiophene-2,3-dione, (b) Compound 2 and (c) Compound 3 obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 4- and 6-MBT.

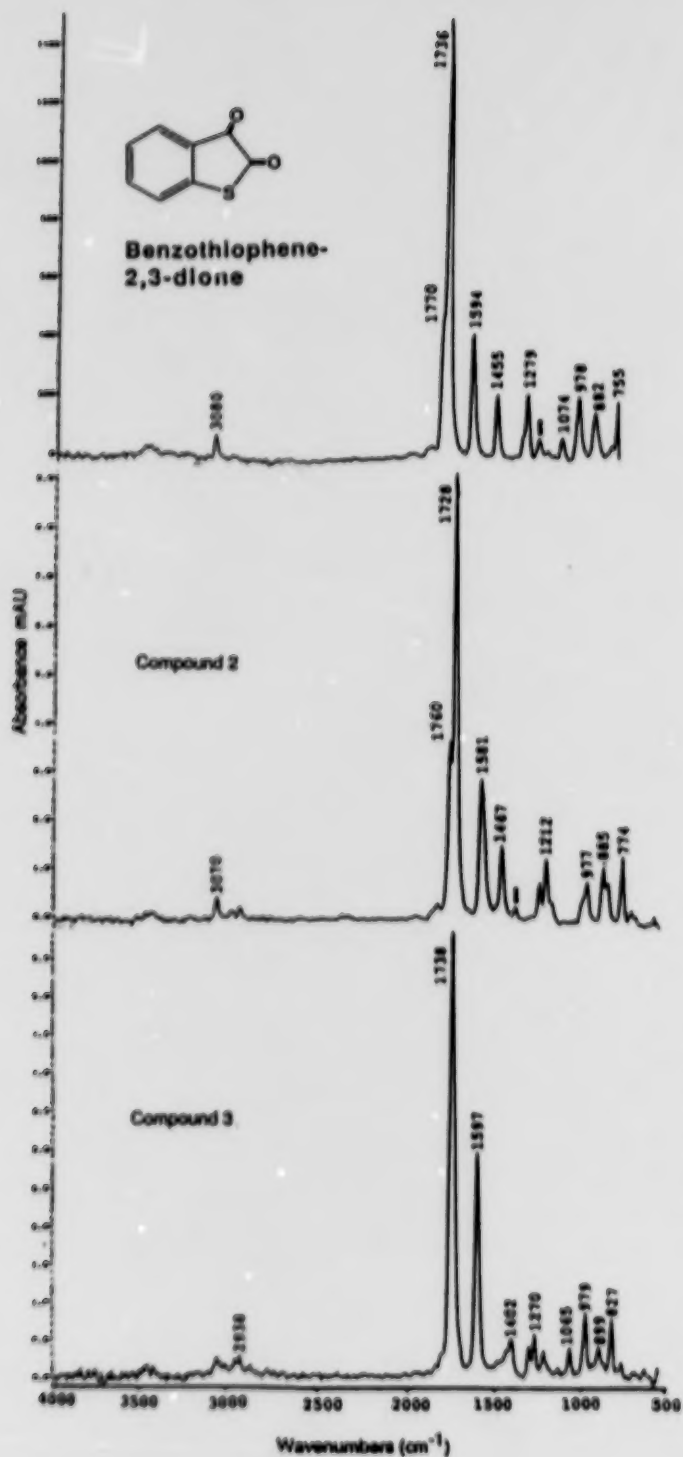


Figure 4.9. FTIR spectra of (a) authentic benzothiophene-2,3-dione, (b) Compound 2 and (c) Compound 3 obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 4- and 6-MBT.

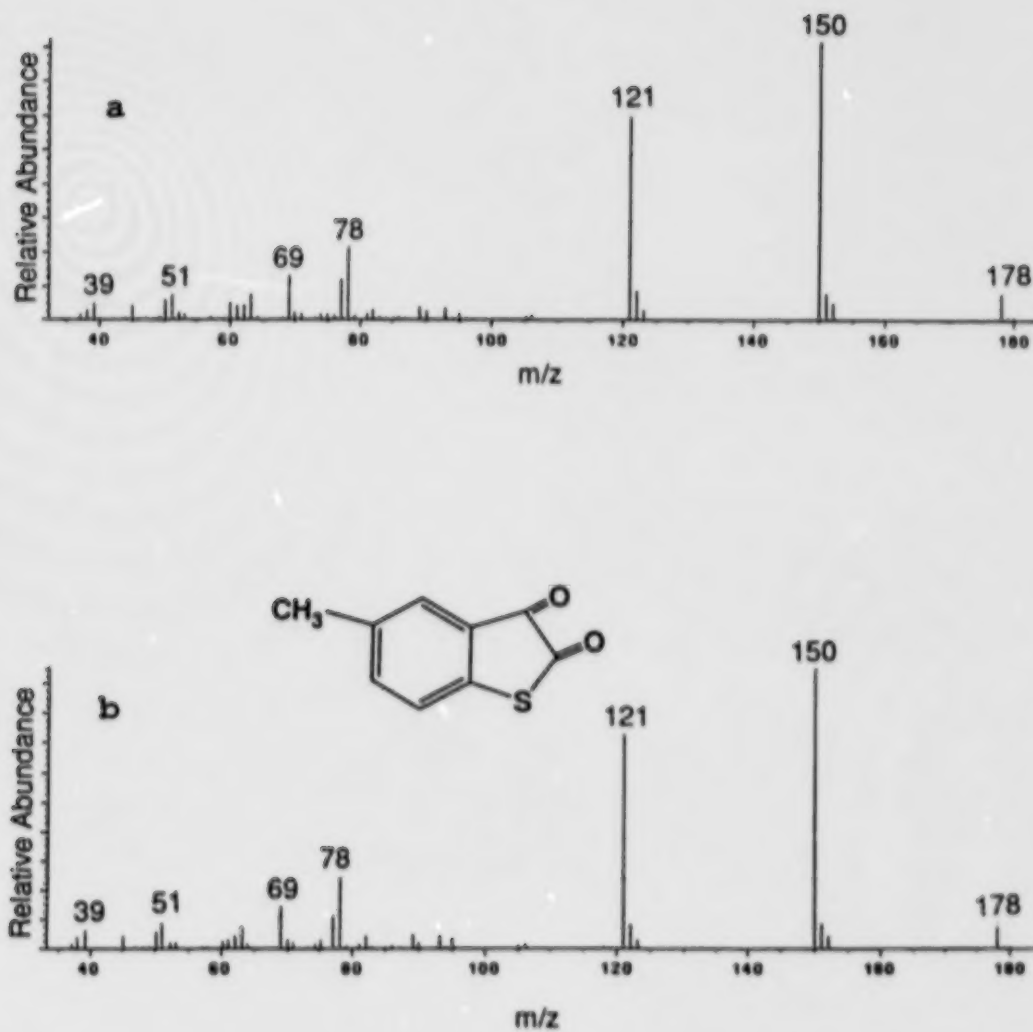


Figure 4.10. Mass spectra of (a) the metabolite obtained from a culture of BT1 grown on 1-MN in the presence of 5-MBT; (b) authentic 5-methylbenzothiophene-2,3-dione.

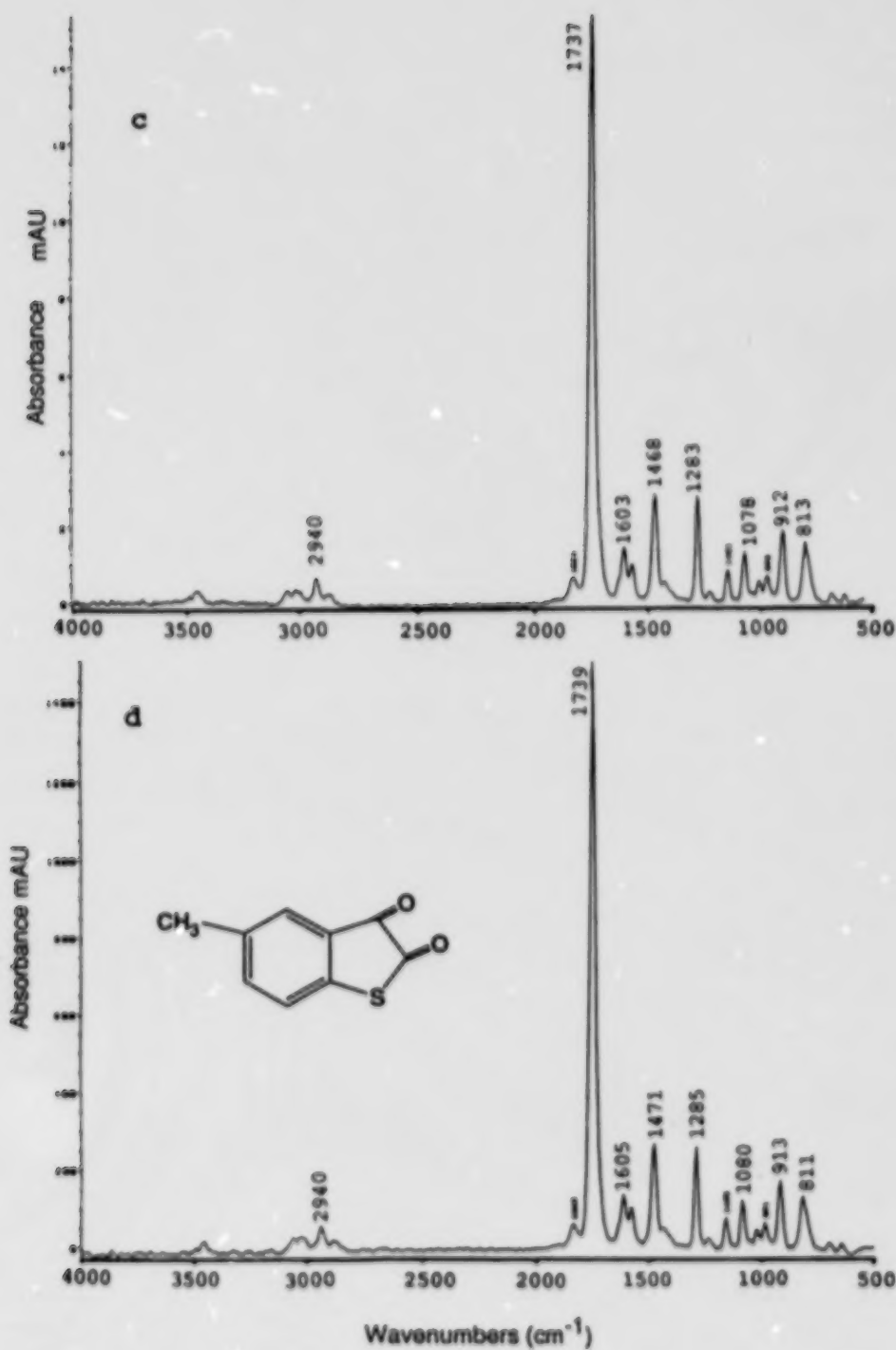


Figure 4.10. FTIR spectrum of (c) the metabolite obtained from a culture of BT1 grown on 1-MN (cont'd) in the presence of 5-MBT and (d) authentic 5-MBT-2,3-dione,

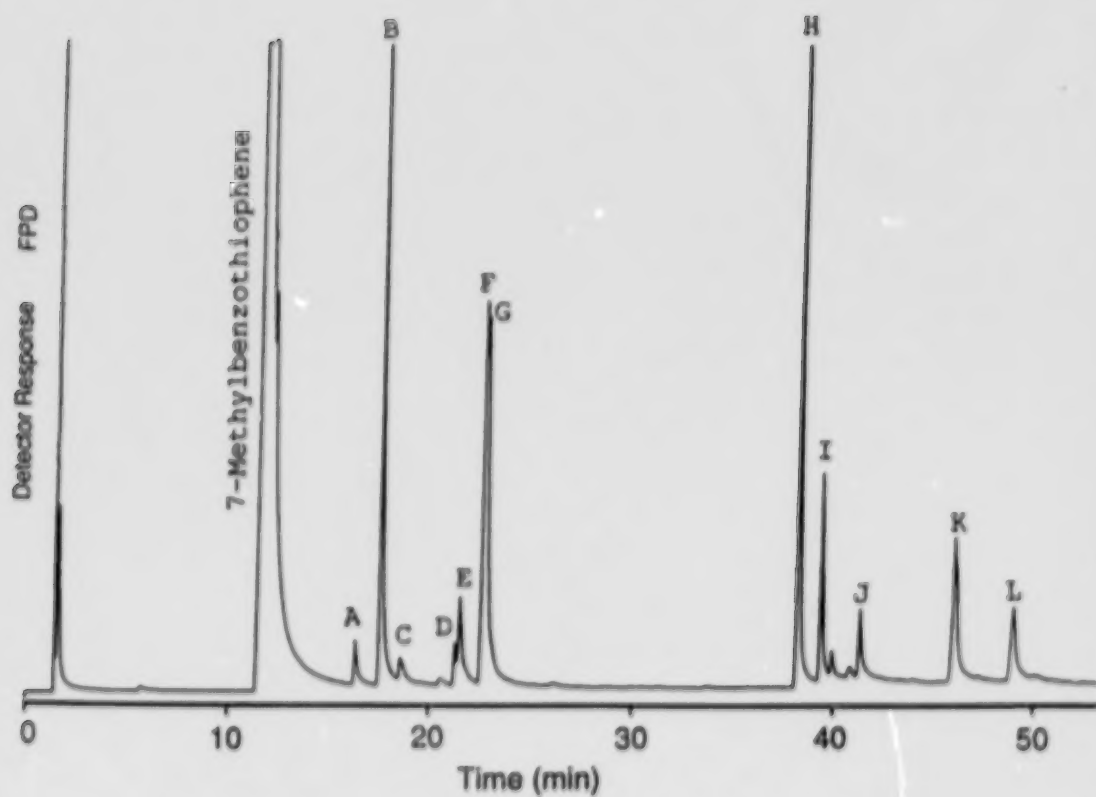


Figure 4.11. GC analysis of the methylene chloride extract of isolate BT1 grown on 1-MN in the presence of 7-MBT.

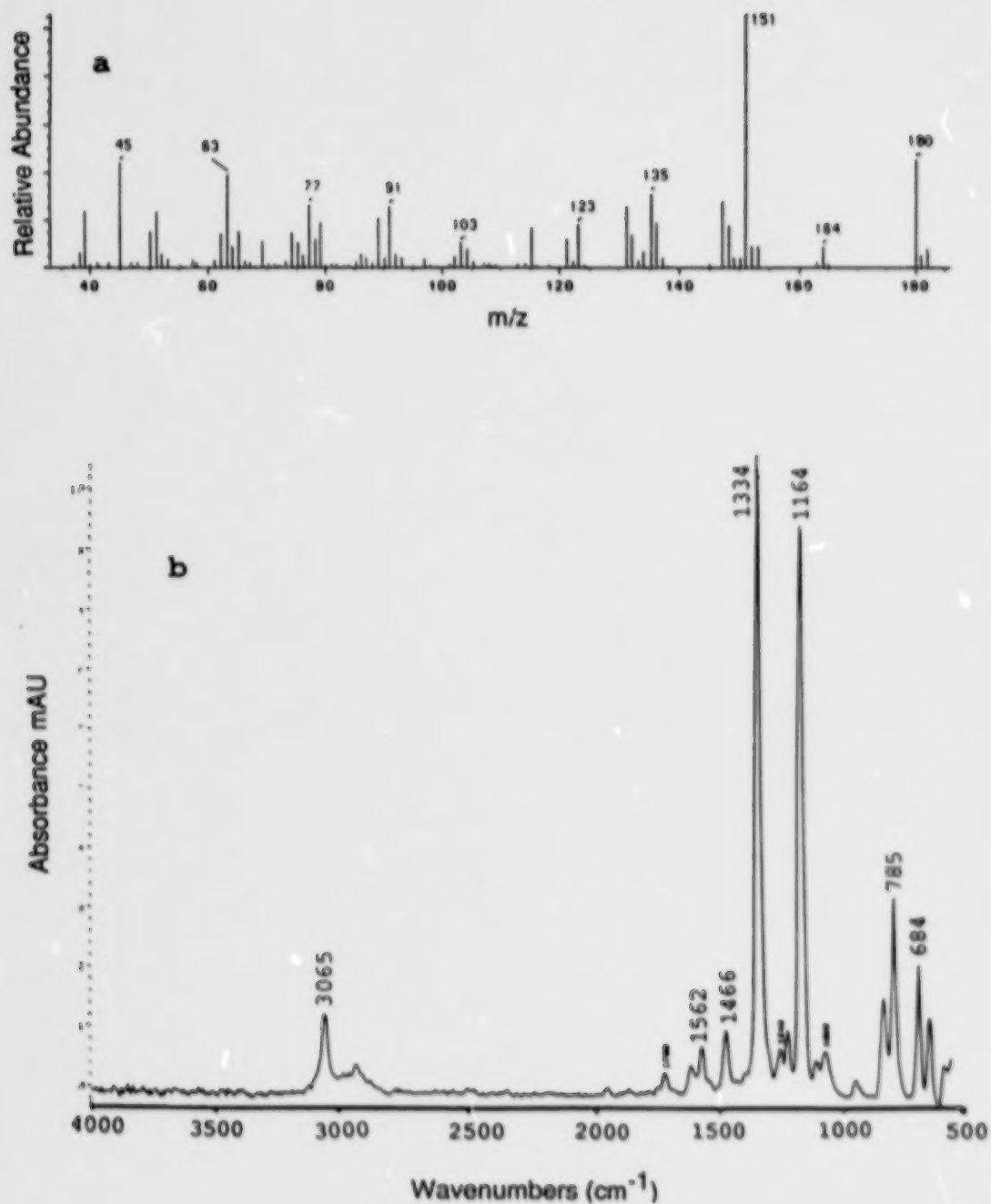


Figure 4.12. Spectra of the Compound G obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 7-MBT; (a) mass spectrum, (b) FTIR spectrum.

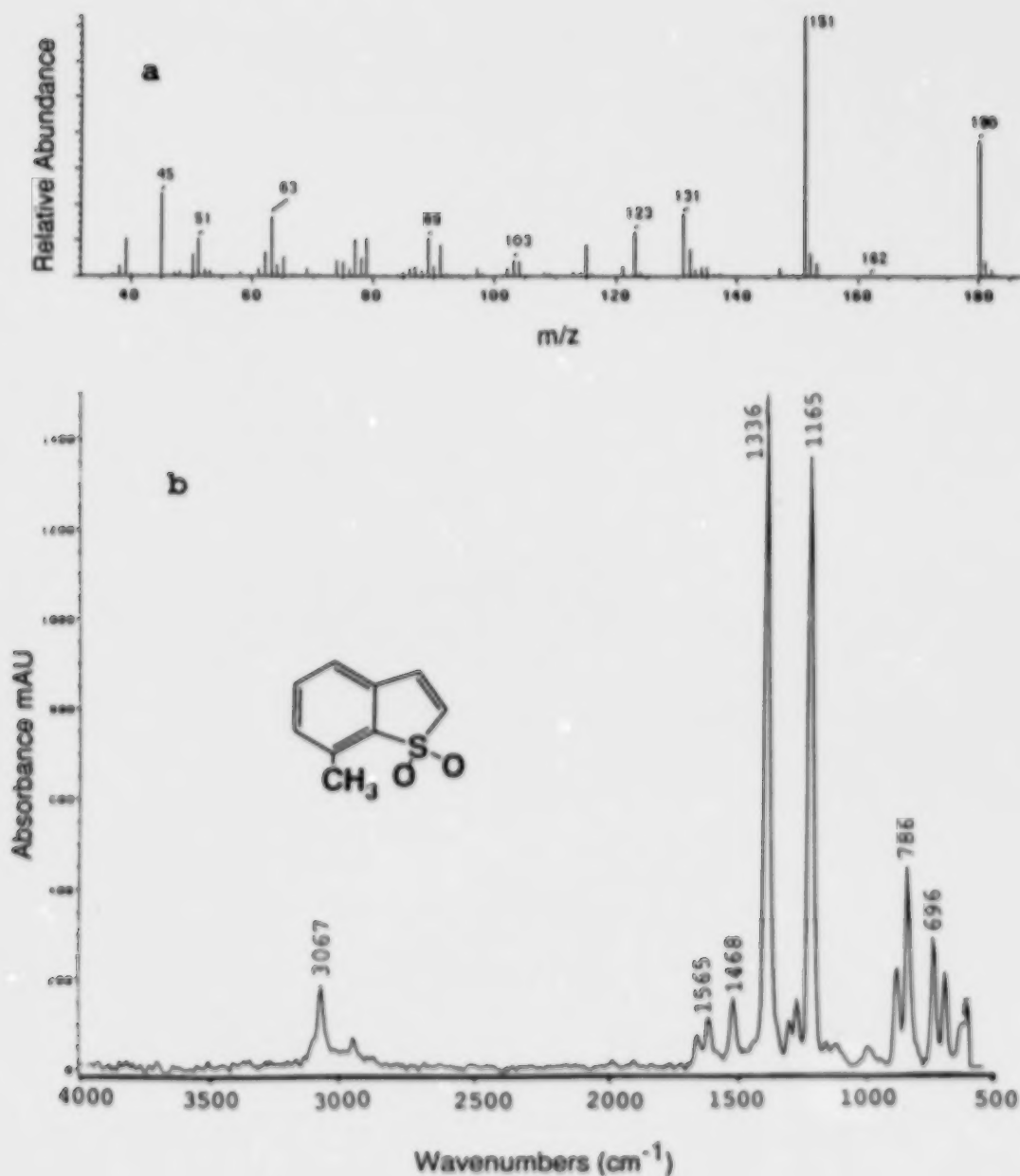


Figure 4.13. Spectra of authentic 7-MBT sulfone; (a) mass spectrum, (b) FTIR spectrum.

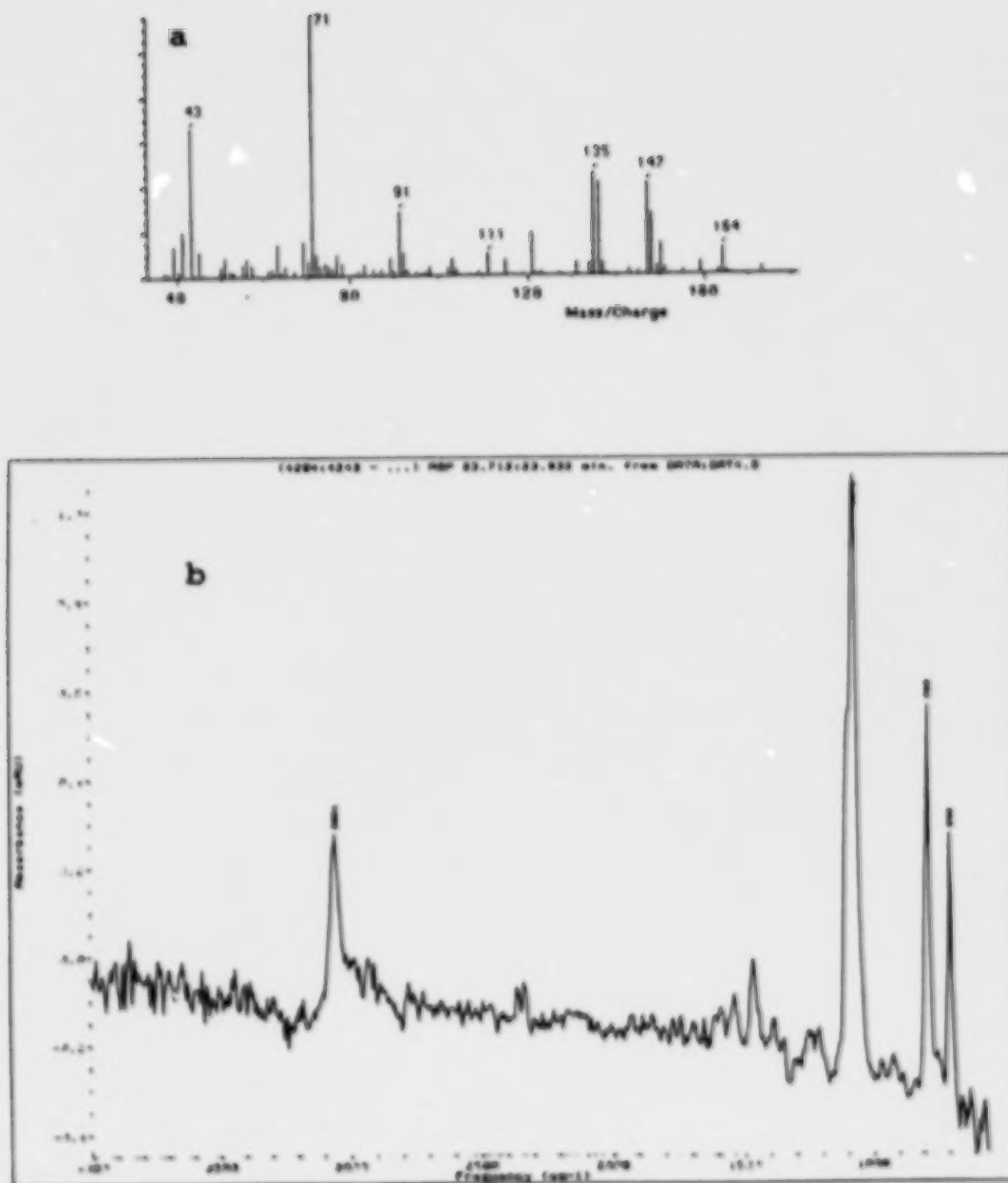


Figure 4.14. Spectra of Compound F obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 7-MBT; (a) mass spectrum, (b) FTIR spectrum.

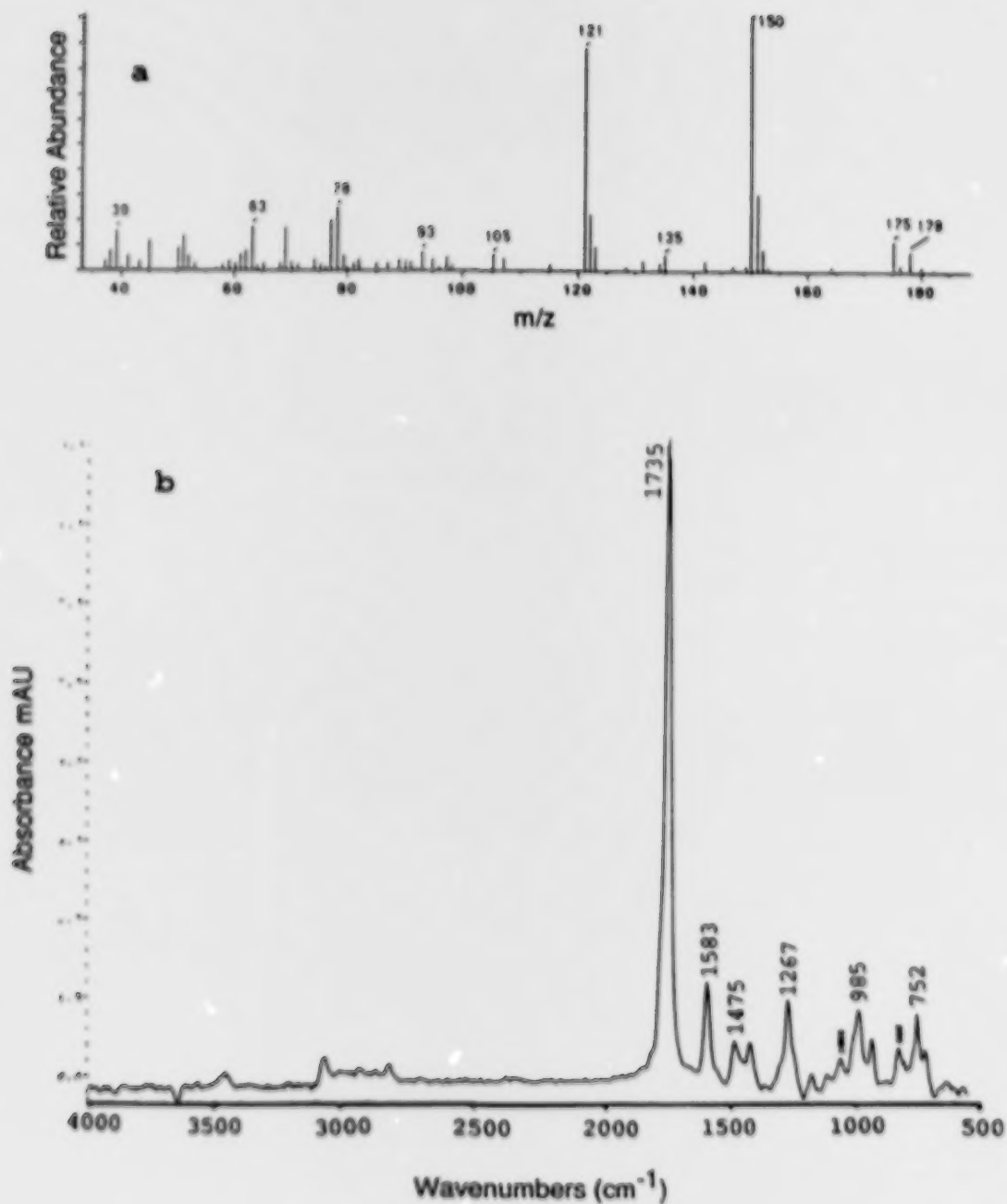


Figure 4.15. Spectra of Compound D obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 7-MBT; (a) mass spectrum, (b) FTIR spectrum.

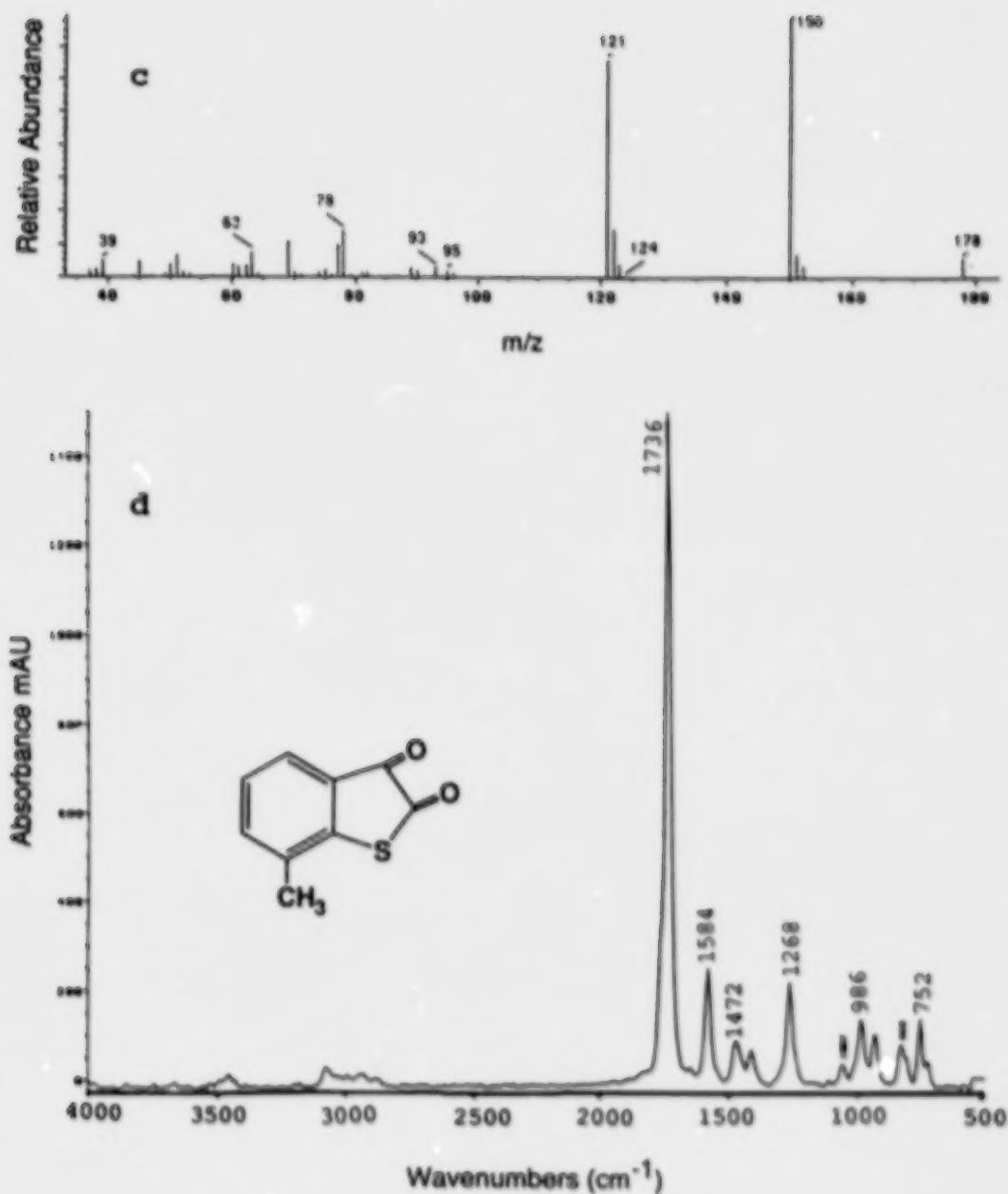


Figure 4.15. Spectra of authentic 7-MBT-2,3-dione; (c) mass spectrum, (d) FTIR spectrum.
(cont'd)

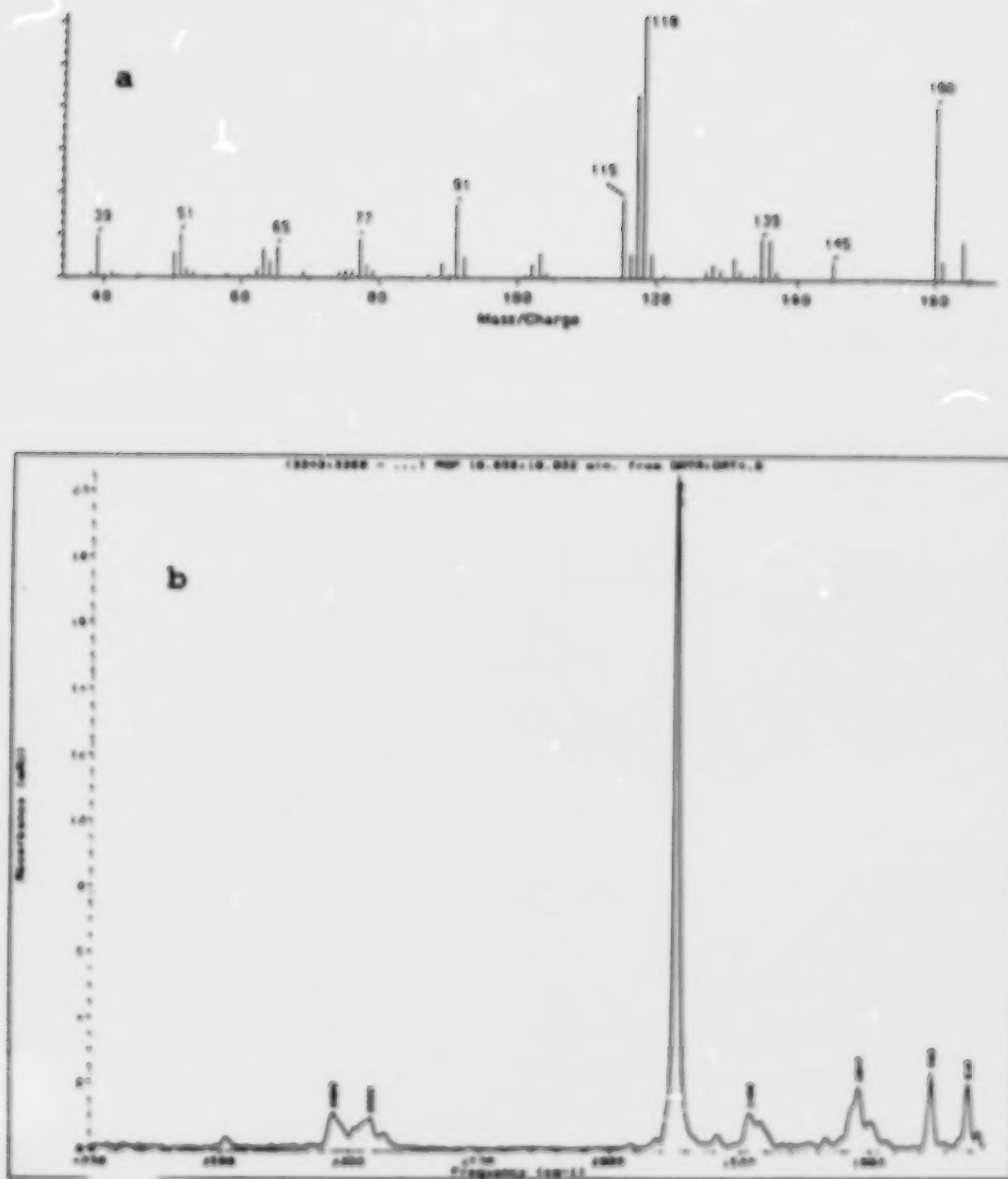


Figure 4.16. Spectra of Compound B obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 7-MBT; (a) mass spectrum, (b) FTIR spectrum.

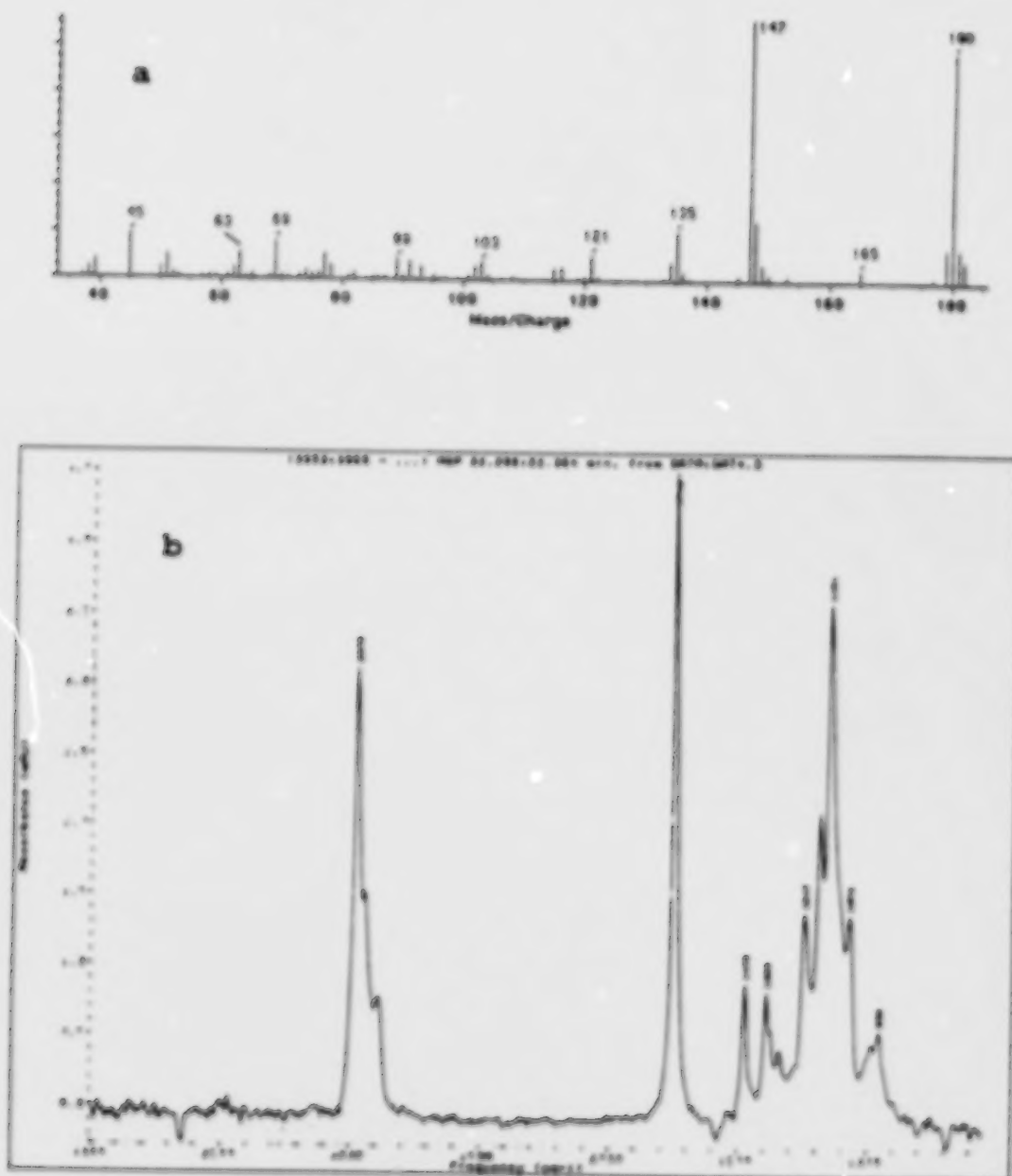


Figure 4.17. Spectra of Compound E obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 7-MBT; (a) mass spectrum, (b) FTIR spectrum.

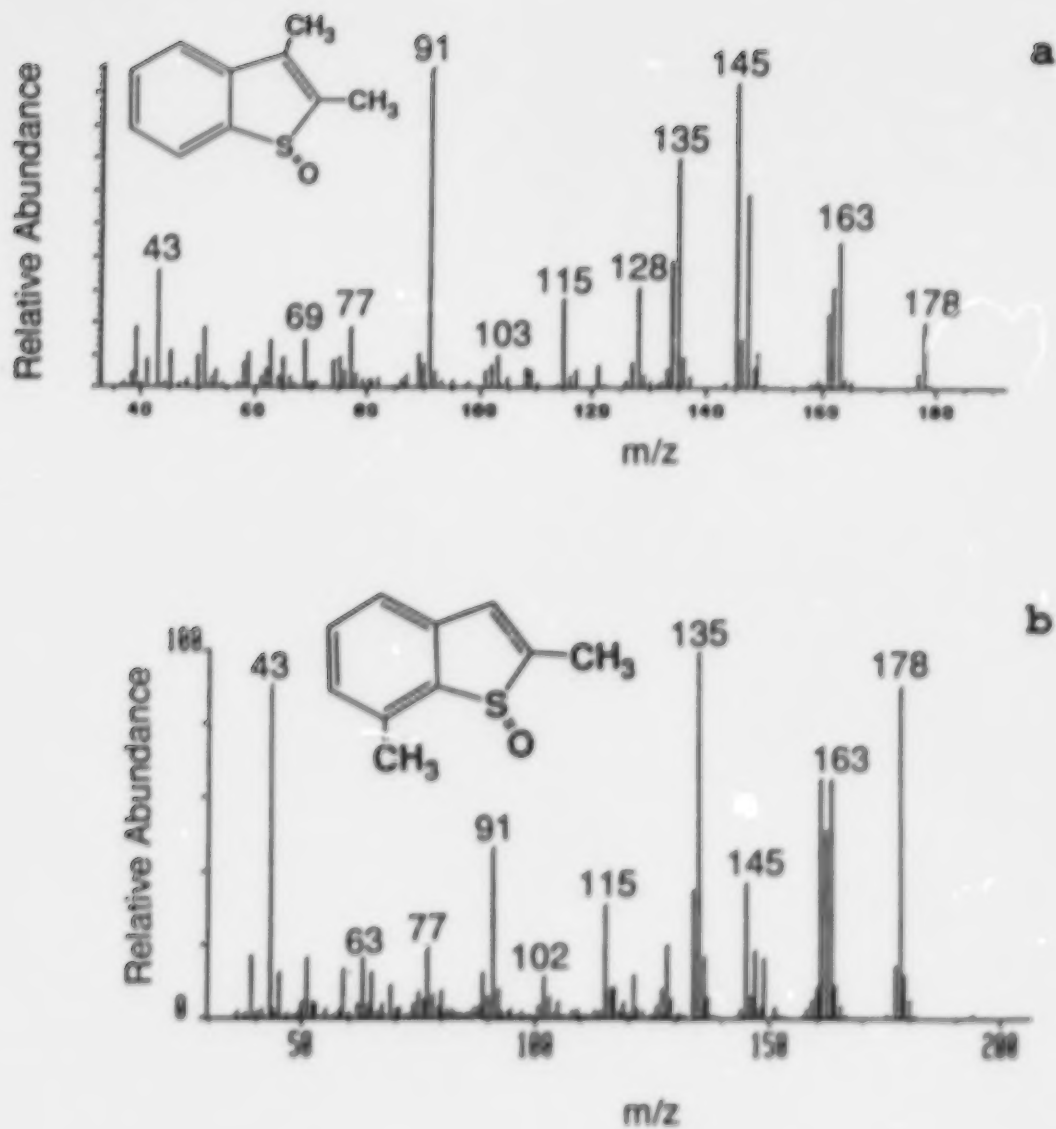


Figure 4.18. Mass spectra of sulfoxides of (a) 2,3-DMBT and (b) 2,7-DMBT that were produced as metabolites by isolate BT1 grown on 1-MN in the presence of 2,3-DMBT or 2,7-DMBT, respectively.

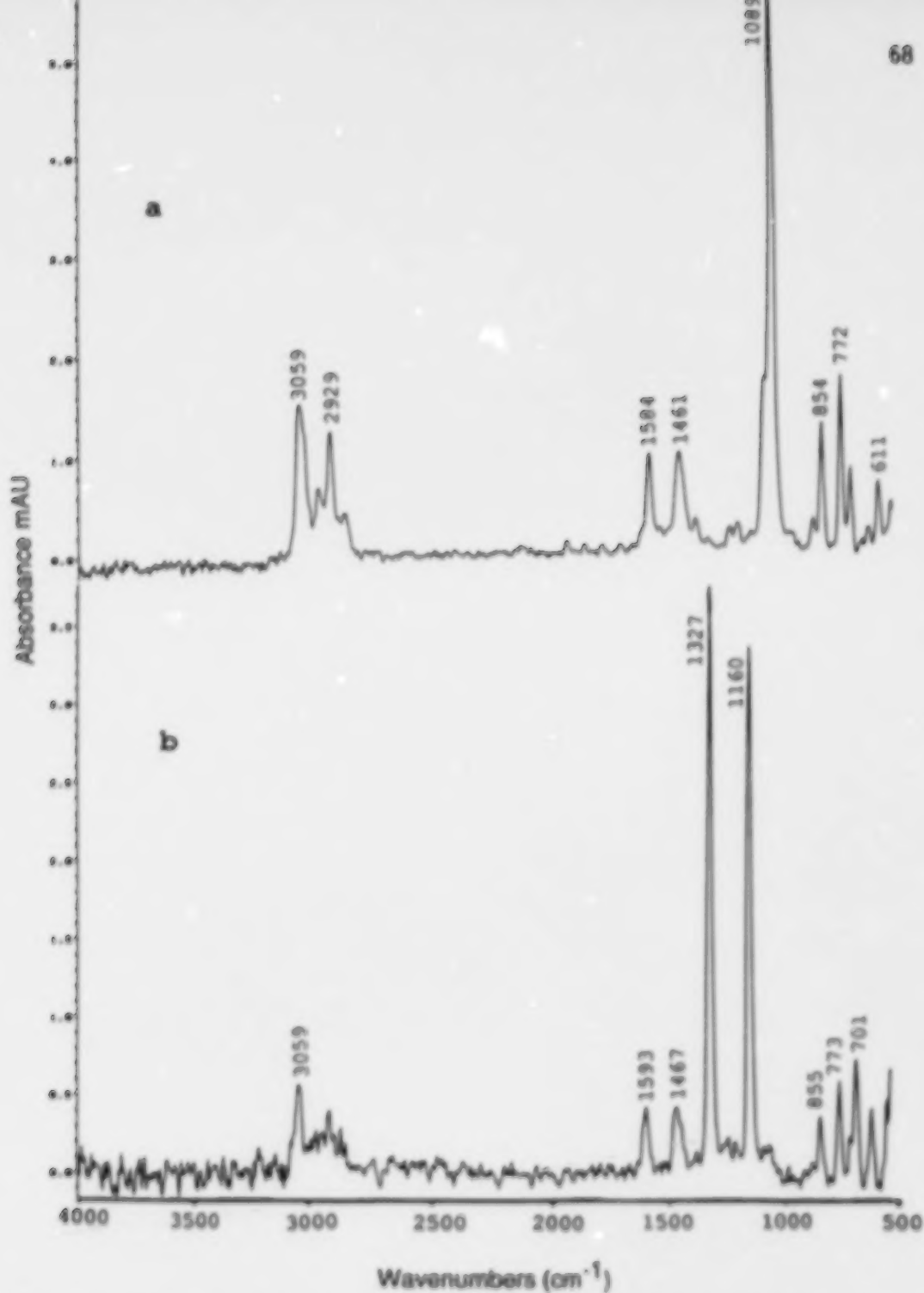


Figure 4.19. FTIR spectra of (a) 2,7-DMBT sulfoxide and (b) 2,7-DMBT sulfone that were produced as metabolites by isolate BT1 grown on 1-MN in the presence of 2,7-DMBT.

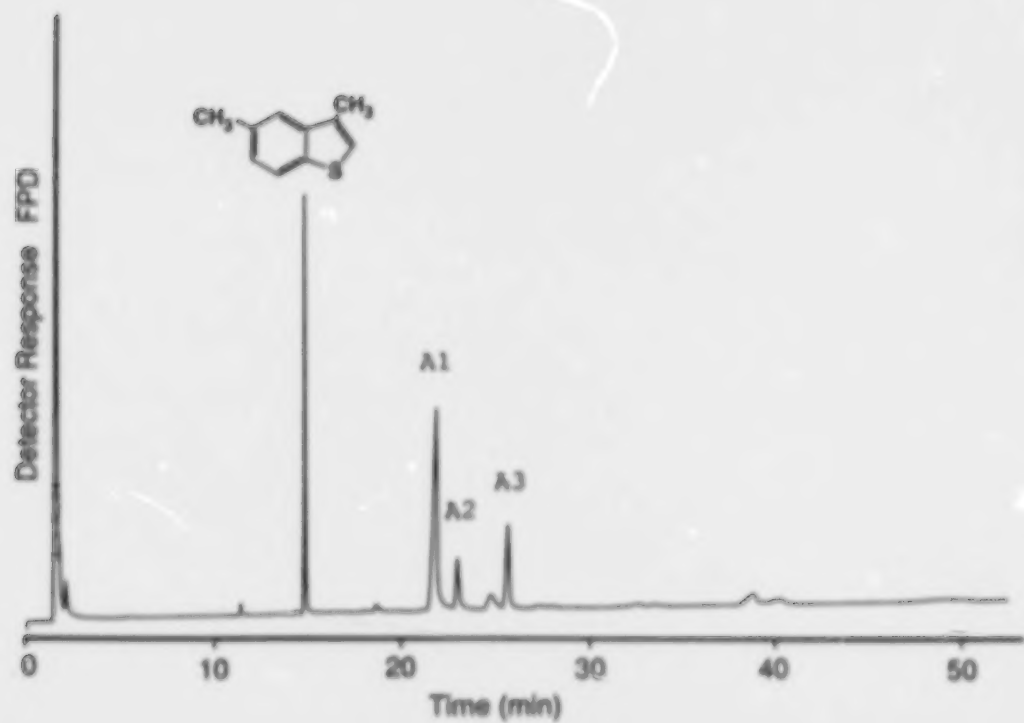


Figure 4.20. GC analysis of the methylene chloride extract of isolate BT1 grown on 1-MN in the presence of 3,5-DMBT.

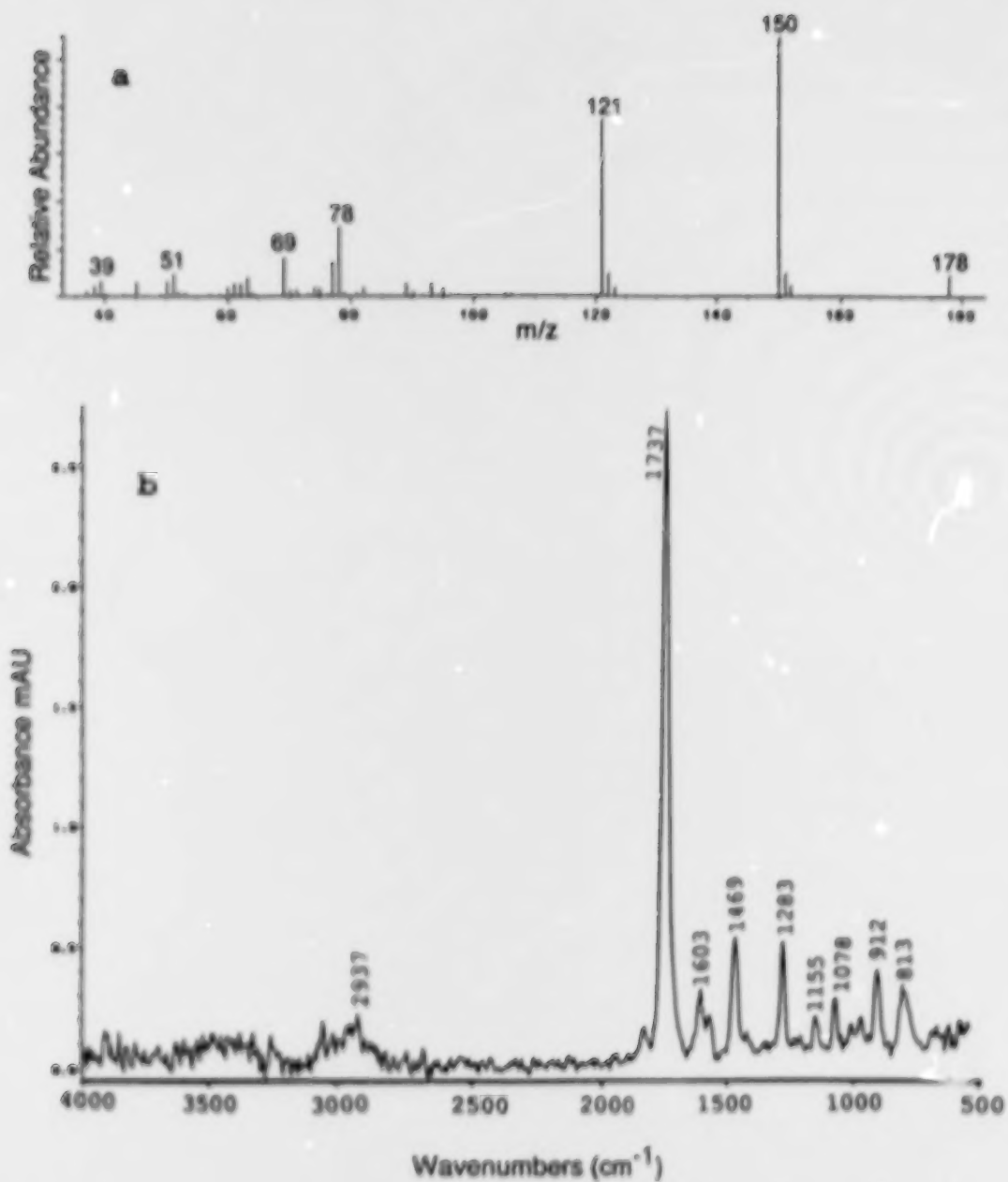


Figure 4.21. Spectra of Compound A1 obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 3,5-DMBT; (a) mass spectrum, (b) FTIR spectrum.

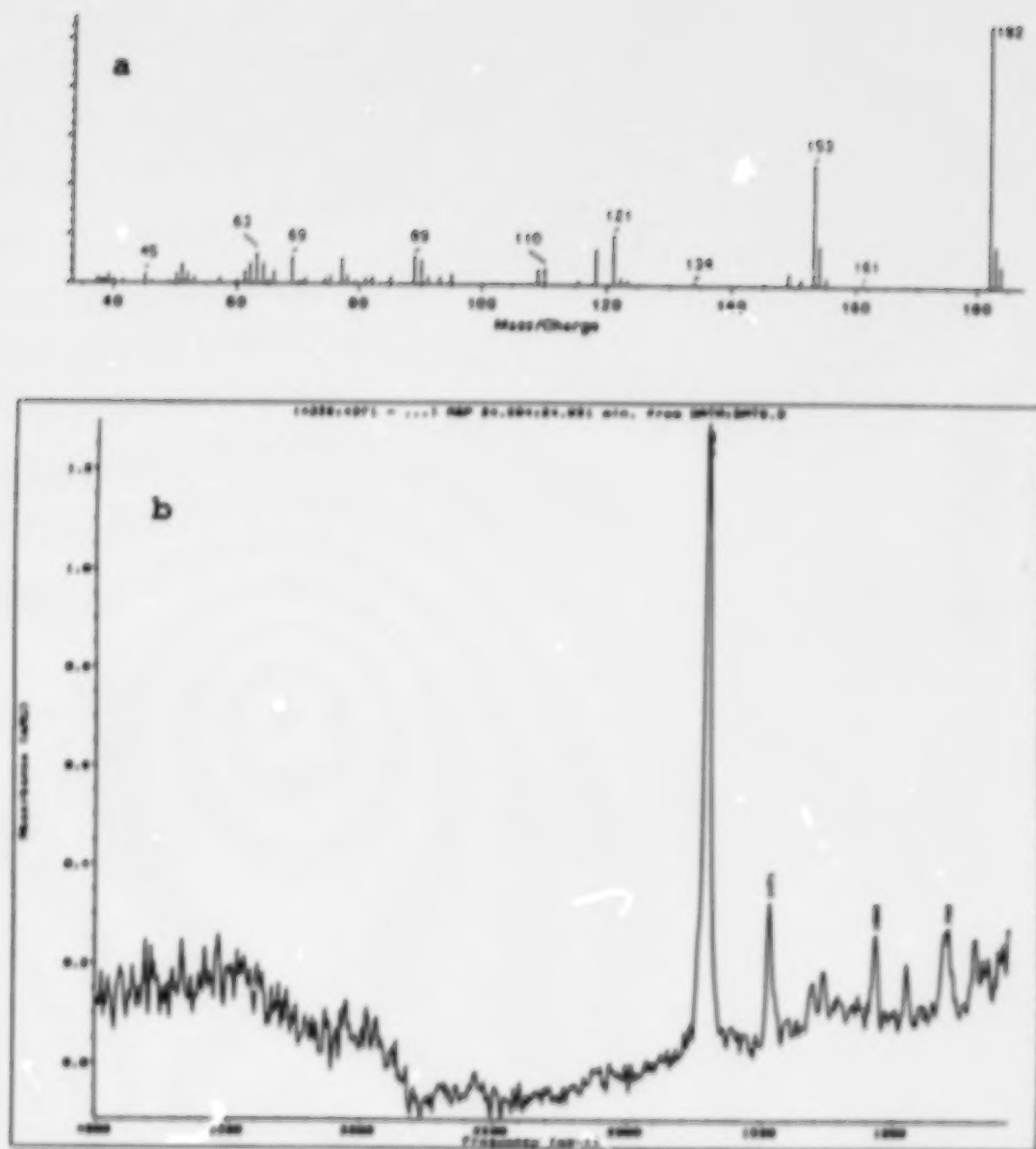


Figure 4.22. Spectra of Compound A2 obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 3,5-DMBT; (a) mass spectrum, (b) FTIR spectrum.

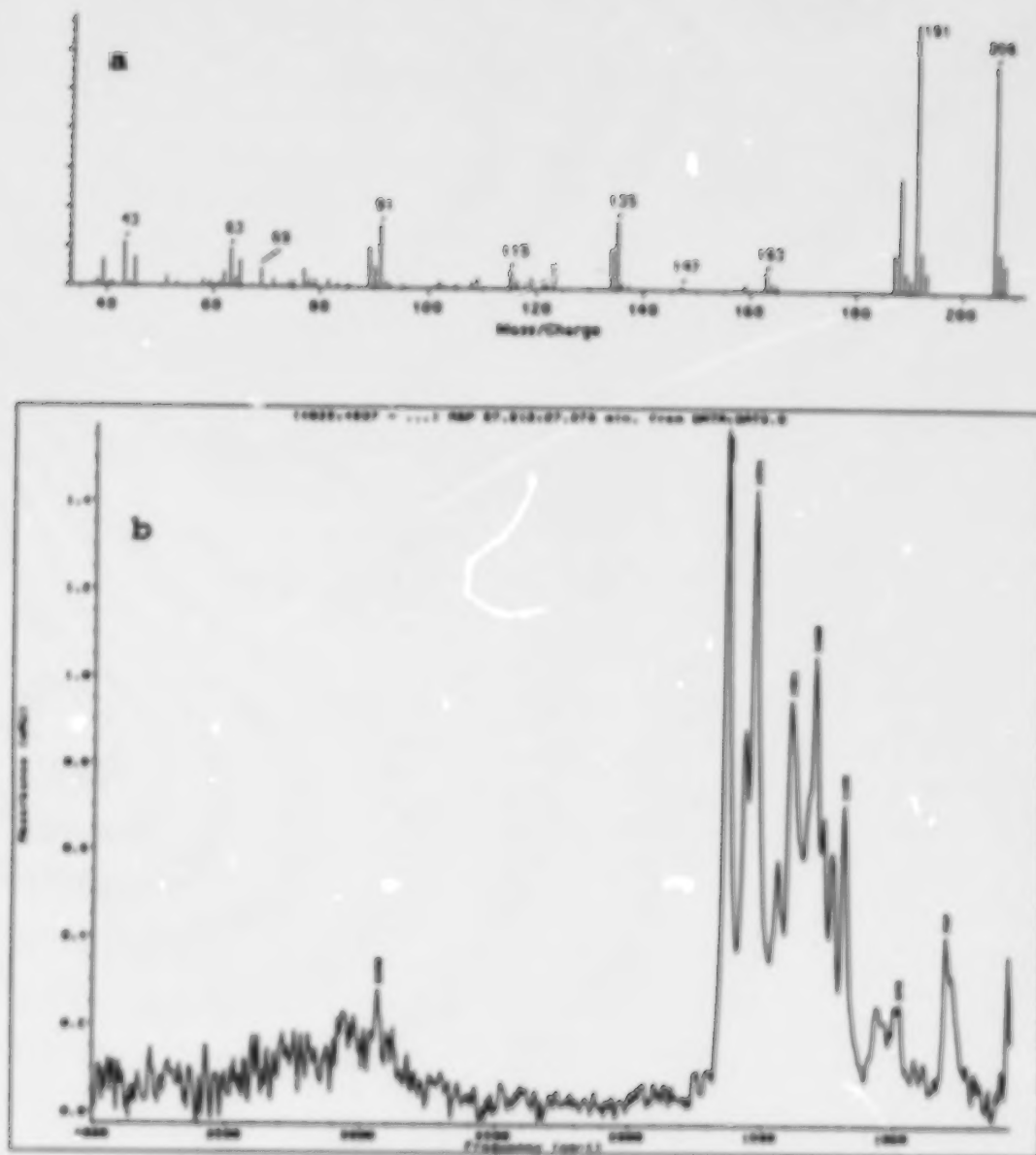


Figure 4.23. Spectra of Compound A3 obtained from a culture of BT1 grown on 1-MN in the presence of a mixture of 3,5-DMBT; (a) mass spectrum, (b) FTIR spectrum.

5.0 RESULTS FROM STUDIES WITH ALKYL DIBENZOTHIOPHENES

There have been numerous studies on dibenzothiophene (DBT) transformations in pure cultures (Hou and Laskin 1976; Kodama 1977; Foght and Westlake 1988) and several intermediates of its cometabolism have been identified (Kodama *et al.* 1970; Kodama *et al.* 1973; Labadie and Gibson, 1977). The major product found is 3-hydroxy-2-formylbenzothiophene (HFBT).

Using a procedure similar to that outlined by Kodama *et al.* (1970) for the isolation of HFBT, we isolated a yellow crystal metabolite from a culture that was metabolizing DBT (unpublished data). GC-MS analysis produced the mass spectrum shown in Figure 5.1a. The metabolite had a molecular weight of 178 which is consistent with it being HFBT. This compound had a melting range of 106-109°C which agrees closely with the literature value of 108°C for 3-hydroxy-2-formylbenzothiophene (Kodama *et al.* 1970). Based on melting point, the metabolite was not the other possible isomer, 2-hydroxy-3-formylbenzothiophene that has a melting point of 130°C (Kodama *et al.* 1970).

The mass spectrum of HFBT shows little fragmentation (Figure 5.1a). The abundant (M-1)⁺ ion at m/z 191 indicates the presence of a labile hydrogen atom (McLafferty 1980). Loss of -CHO would yield the ion at m/z 163. The subsequent loss of -OH would yield the ion at m/z 146. The ion at m/z 121 (M-57)⁺ is likely C₇H₅S which was observed by Porter (1967) in his studies of the mass spectra of 2-MBT and 3-MBT. This ion was also observed in Figures 4.4a and 4.4b. The FTIR spectrum of HFBT is shown in Figure 5.1b.

Analyses of biodegraded petroleum have indicated that methyl dibenzothiophenes can be metabolized by microorganisms. However, only the results of a preliminary investigation of 4-methyldibenzothiophene (4-MDBT) transformation by *Pseudomonas* strain HL7b have been reported (Fedorak 1990). For that study, 4-MBT was synthesized from DBT and, based on GC analysis, the product was a mixture of 71% 4-MDBT and 29% unreacted DBT. Analysis of the metabolites formed by strain HL7b showed two sulfur-containing compounds. GC-MS analysis indicated that one was HFBT and the other gave a molecular ion m/z 192 which is 14 mass units greater than HFBT. This mass spectrum of the second compound is shown in Figure 5.2 and it is consistent with this metabolite being methyl-HFBT. This suggested that the unsubstituted ring was preferentially oxidized and is in agreement with studies on the bacterial metabolism of other methyl-substituted aromatic hydrocarbons such as 1-methylnaphthalene (Dean-Raymond and Bartha 1975), 2-methylnaphthalene (Williams *et al.* 1975), and 3-methyl- and 4-methylbiphenyls (Fedorak and Westlake 1983a).

Based on GC analysis of the products of strain HL7b transformation of the mixture of DBT and 4-MDBT (Fedorak 1990), HFBT accounted for 58% of the two products recovered and the methyl-HFBT accounted for 42%. This proportion was quite different from that of the added

substrates suggesting that (1) the methyl-substituted compound was less susceptible to the biotransformation or (2) both the methylated ring and the non-methylated benzene ring of 4-MBT were susceptible to cleavage yielding a mixture of HFBT and methyl-HFBT from this one substrate.

The focus of this project was to study the microbial transformations of high-purity methyl-dibenzothiophenes to resolve whether there is a preference toward oxidizing the non-methylated ring and to determine whether the same trend is observed with different methyl isomers. Two bacterial isolates were used for this work. One was *Pseudomonas* strain BT1 (Fedorak and Grbic-Galic 1991) which was used in the work with the alkyl benzothiophenes (section 4.0). The isolation and characteristics of the other bacterium, designated strain W1, are detailed in section 5.1. Because the biotransformations of the DBTs was thought to be the result of cometabolism, the isolates were incubated with 1-MN in the presence of one of the sulfur heterocycles. For the initial experiments, the cultures were incubated for 14 days. The medium often became purple but few metabolites were detected when the extract was analyzed by GC. It was presumed that dimerization of products might have occurred as has been reported for HFBT dimerization in DBT-degrading cultures (Kodama *et al.* 1970). These dimers would not be found using our GC methods. Thus in many cases, cultures were incubated for only 2 days to reduce the chance of dimer production and thereby improve the likelihood of finding monomers.

5.1 Isolation and characteristics of strain W1

Strain W1 was isolated from an enrichment culture inoculated with water from a tropical freshwater aquarium. The enrichment was grown in mineral medium on 1-MN and DBT. Isolation was done using standard plating techniques. Strain W1 was a short, Gram-negative rod that grew well in liquid medium containing 1-MN and DBT, turning the medium orange and subsequently purple. The isolate could also be maintained on 1-MN. Experience showed that frequent transfers of W1 (e.g. every 3 to 4 days) into fresh medium with 1-MN and DBT would maintain an active culture. These transfers were done before the medium turned purple. W1 would not survive intervals of 2 weeks between transfers. When W1 was incubated with 1-MN, the major product was 1-naphthoic acid which was identified by GC-MS and comparison with an authentic standard. Two intermediates were also detected by GC-MS. These were 1-naphthalenemethanol and 1-naphthaldehyde. The mechanisms by which 1-MN was metabolized by isolates W1 and BT1 were different. W1 preferentially oxidized the methyl group yielding 1-naphthoic acid, whereas BT1 attacked the unsubstituted ring producing 3-methylsalicylic acid as the major, transient intermediate (Fedorak and Grbic-Galic 1991).

The ability of isolate W1 to mineralize several different hydrocarbons was tested using the method of Foght *et al.* (1990). Replicate cultures were established with Norman Wells crude oil

spiked with each of the ^{14}C -labeled substrates. After 2 weeks incubation, the cultures were acidified and flushed to recovery any $^{14}\text{CO}_2$ that had been produced. The substrates and the percent of the original label recovered as $^{14}\text{CO}_2$ were: hexadecane, 4.2%; naphthalene, 56%; biphenyl, 4.3%; phenanthrene, 11%; and anthracene, 0.9%. Using the criterion that significant mineralization occurred when >5% of the label was recovered as $^{14}\text{CO}_2$ (Foght *et al.* 1990), only naphthalene and phenanthrene were used by isolate W1.

5.2 Studies with isolate BT1

Isolate BT1 was maintained in liquid culture on 1-MN and, unless otherwise noted, the inocula used in the experiments described below originated from this maintenance culture. Surprisingly, isolate BT1 survived repeated transfers in liquid medium containing DBT as the sole carbon and energy source. The major metabolite found in the spend culture medium was 2HBT. Although the cell densities in these cultures were always low, portions were used as inocula in a few of the experiments with methylbenzothiohenes.

5.2.1 Cometabolism of DBT

Figure 5.3a shows the mass spectrum of the major sulfur-containing metabolite found in the extract of isolate BT1 grown for 2 days in the presence of DBT. The molecular ion appeared to be m/z 164 and the parent ion was at m/z 136. Other abundant ions were at m/z 108, 69 and 50. This mass spectrum was very similar to that of authentic benzothiophene-2,3-dione shown in Figure 4.8a. The FTIR spectrum of the metabolite from DBT is shown in Figure 5.3b. This spectrum matched that of authentic benzothiophene-2,3-dione (Figure 4.9a). Clearly the metabolite was benzothiophene-2,3-dione.

5.2.2 Cometabolism of 2-methylbenzothiophene

The GC-FPD analysis of the extract from a 2-day-old culture of isolate BT1 grown on 1-MN and 4-methylbenzothiophene showed one major and one minor peak. The former was about 10 times larger than the latter. The mass spectrum of the major metabolite is shown in Figure 5.4 and it matched the spectrum of authentic 5-methylbenzothiophene-2,3-dione shown in Figure 4.10b. The mass spectrum of the minor metabolite is shown in Figure 5.5 and it was nearly identical to that of authentic benzothiophene-2,3-dione (Figure 4.8a).

5.2.3 Cometabolism of 3-methylbenzothiophene

Two abundant sulfur-containing compounds were observed in the extract from isolate BT1

grown on 1-MN in the presence of 3-methyldibenzothiophene. Figure 5.6a shows the mass spectrum of the more abundant metabolite and it is very similar to that of the metabolite from 6-MBT (Compound 3, Figure 4.8c). Thus the metabolite was 6-methylbenzothiophene-2,3-dione.

The second most abundant metabolite from 3-methyldibenzothiophene had a retention time of 11.72 min and the mass spectrum of this compound is shown in Figure 5.6b. The strong molecular ion at m/z 148 and the abundant $(M-1)^+$ ion at m/z 147 were consistent with the compound being 6-MBT and this spectrum closely matched that of the authentic 6-MBT sample (Figure 5.6c).

The culture of isolate BT1 maintained on DBT was used to inoculate medium containing 3-methyldibenzothiophene. GC-MS analysis of the extract from this culture that had incubated for 2 days showed a major metabolite peak with a strong molecular ion at m/z 192 (Figure 5.7). The fragmentation pattern of this metabolite was similar to that of the methyl-HFBT shown in Figure 5.2), and suggested that it was 6-methyl-3-hydroxy-2-formylbenzothiophene. The FTIR spectrum of this metabolite is shown in Figure 5.7b. Computer matching of this spectrum and that of HFBT (Figure 5.1b) showed they had 92% similarity.

5.2.4 Cometabolism of 1- and 3-methyldibenzothiophene

The synthesis used for 1-methyldibenzothiophene gave a mixture of this and the 3-methyl isomer. Based on the FID response from GC analysis of this mixture, it contained approximately 48% of the 1-methyl isomer and 52% of the 3-methyl isomer.

In the extract from a 2-day-old culture of isolate BT1 grown on 1-MN plus the mixture of these two isomers, there was an abundant sulfur-containing compound with a retention time of 11.74 min. It had a molecular ion of m/z 148 and its mass spectrum matched that of a methylbenzothiophene. Two other sulfur-containing metabolites were present and approximately the same concentration and their mass spectra are shown in Figure 5.8. These spectra are very similar and match those of Compounds 2 and 3 in Figure 4.8. Thus the metabolites from the mixture of methyldibenzothiophenes were 6-methylbenzothiophene-2,3-dione and 4-methylbenzothiophene-2,3-dione. The former compound arose from 3-methyldibenzothiophene and the latter from 1-methyldibenzothiophene. The later eluting peaking, corresponding to 6-methylbenzothiophene-2,3-dione, had the same GC retention time and mass spectrum as the dione from the culture that received pure 3-methyldibenzothiophene (Figure 5.6a).

5.2.5 Cometabolism of 4-methyldibenzothiophene

The GC analysis of an extract of a 14-day-old culture BT1 grown on 1-MN and 4-methyldibenzothiophene showed two sulfur-containing metabolites. The mass spectrum of the more abundant metabolite had a weak molecular ion at m/z 178 and two strong fragment ions at m/z 150 and m/z

121. The fragmentation pattern was essentially the same as that 7-methylbenzothiophene-2,3-dione (Figure 4.15c). The mass spectrum of the minor metabolite showed a strong molecular ion at m/z 162, a small fragment peak at m/z 146 followed by a strong fragment peak at 134 (Figure 5.5). The identity of this compound has not been determined.

The GC analysis of the extract of a 2-day-old culture BT1 grown on 1-MN and 4-methyldibenzothiophene showed two abundant sulfur-containing metabolites that were different from those mentioned in the preceding paragraph. One metabolite had a retention time of 11.73 min and the mass spectrum matched that of a methylbenzothiophene. The mass spectrum of the second metabolite showed that the parent peak was the molecular ion at m/z 192 (Figure 5.9). The fragmentation pattern was very similar to that in Figure 5.7a suggesting that the metabolite was 7-methyl-3-hydroxy-2-formylbenzothiophene. In addition, the GC-MS analysis of the extract from this culture detected a small amount of 7-methylbenzothiophene-2,3-dione.

5.3 Studies with isolate W1

Because of difficulties growing and obtaining reproducible inocula of isolate W1, fewer results were obtained with this bacterium than with isolate BT1. Nonetheless, the ability of isolate W1 to cometabolize some of the methyldibenzothiophenes was demonstrated.

5.3.1 Cometabolism of 2-methyldibenzothiophene

Isolate W1 grown on 1-MN and 2-methyldibenzothiophene on two occasions. The GC analysis of the culture extract from the first experiment showed two sulfur-containing metabolites at retention times 22.75 and 37 min. The GC-MS analysis of the metabolite with the retention time 22.75 showed it was a very minor component and that it was poorly resolved from a metabolite from 1-MN. The mass spectrum of the sulfur-containing metabolite contained many contaminating fragment ions. However, the mass spectrum showed a strong molecular ion at m/z 192, an abundant $(M-1)^+$ ion and small fragment ions at m/z 163, 135, 91, similar to Figure 5.7a. This metabolite was presumed to be 5-methyl-3-hydroxy-2-formylbenzothiophene. The second metabolite, which was detected by GC analysis with the sulfur-specific detector, could not be observed during the GC-MS analysis.

The GC-FFD chromatogram from the second experiment showed several small sulfur-containing peaks. One of them, with the retention time 21.26 min was identified as 5-methylbenzothiophene-2,3-dione. The mass and FTIR spectra of this metabolite are shown in Figure 5.10. These are virtually identical to those of the authentic 5-methylbenzothiophene-2,3-dione in Figure 4.10.

5.3.2 Cometabolism of 4-methyldibenzothiophene

GC analysis of methylene chloride extracts of isolate W1 grown on 1-MN and 4-methyldibenzothiophene showed two sulfur-containing metabolites. A major metabolite with the retention time 22.27 min and a minor metabolite peak at 20.59 min. The mass spectrum of the major metabolite (Figure 5.11) showed a strong molecular ion at m/z 192 and an abundant $(M-1)^+$ ion. The small fragment ions at m/z 163, 135, 121, 91 and 63 were consistent with the metabolite being 7-methyl-3-hydroxy-2-formylbenzothiophene. GC-MS analysis of the minor metabolite showed a weak molecular ion at m/z 178 and two strong fragment peaks at m/z 150 and 121 (Figure 5.12a) consistent with the metabolite being 7-methylbenzothiophene-2,3-dione. The FTIR spectrum of this metabolite (Figure 5.12b) matched that of authentic 7-methylbenzothiophene-2,3-dione (Figure 4.15d).

5.4 Summary

Undoubtedly there were metabolites produced by biotransformations of the methyldibenzothiophenes which were not detected by the GC methods used in this preliminary study. For example, some of the cultures turned purple which suggested that the dimers of the methylhydroxyformylbenzothiophenes were formed. These compounds are not amenable to GC analysis. In addition, thin layer chromatography of some of the culture extracts showed many colored compounds and it is unlikely that all of these were found by the GC analyses. Nonetheless, several cometabolites from the methyldibenzothiophenes were identified.

The data in Table 5.1 summarize the results of the methyldibenzothiophene cometabolism studies with isolates BT1 and W1. In all cases, the corresponding methylbenzothiophene-2,3-dione was produced from each isomer of methyldibenzothiophene. In addition, benzothiophene-2,3-dione was also produced from dibenzothiophene by isolate BT1. Methylhydroxyformylbenzothiophenes were detected as products of the cometabolism of three of the four methyldibenzothiophene isomers. Although a trace amount of benzothiophene-2,3-dione was detected in a culture of BT1 grown in the presence of 2-methyldibenzothiophene (section 5.2.2), the results obtained in this study clearly show that the unsubstituted ring was preferentially cleaved. The prevalence of the 2,3-diones is somewhat unique because among the numerous studies on the microbial metabolism of dibenzothiophene, only Bohannon *et al.* (1977) have reported benzothiophene-2,3-dione as a metabolite.

Methylbenzothiophenes were frequently detected in cultures of BT1 and W1 grown in the presence of different methyldibenzothiophenes. There are no reports in the literature of the microbial formations of benzothiophene from DBT and the origins of the methylbenzothiophenes in our cultures are unknown.

Table 5.1 Products of the cometabolism of methyl dibenzothiophenes by isolates BT1 and W1.

Dibenzothiophene	Methyl BT ^a -2,3-dione		Methyl-HFBT ^a		Methyl-BT ^b	
	BT1	W1	BT1	W1	BT1	W1
1-Methyl-	Present	NT ^c	ND ^d	NT	Present	NT
2-Methyl-	Present	Present	ND	Present	Present	Present
3-Methyl-	Present	NT	Present	NT	Present	NT
4-Methyl-	Present	Present	Present	Present	Present	Present

^a HFBT = Hydroxyformylbenzothiophene

^b BT = Benzothiophene

^c NT = Not tested adequately to judge

^d ND = Not detected

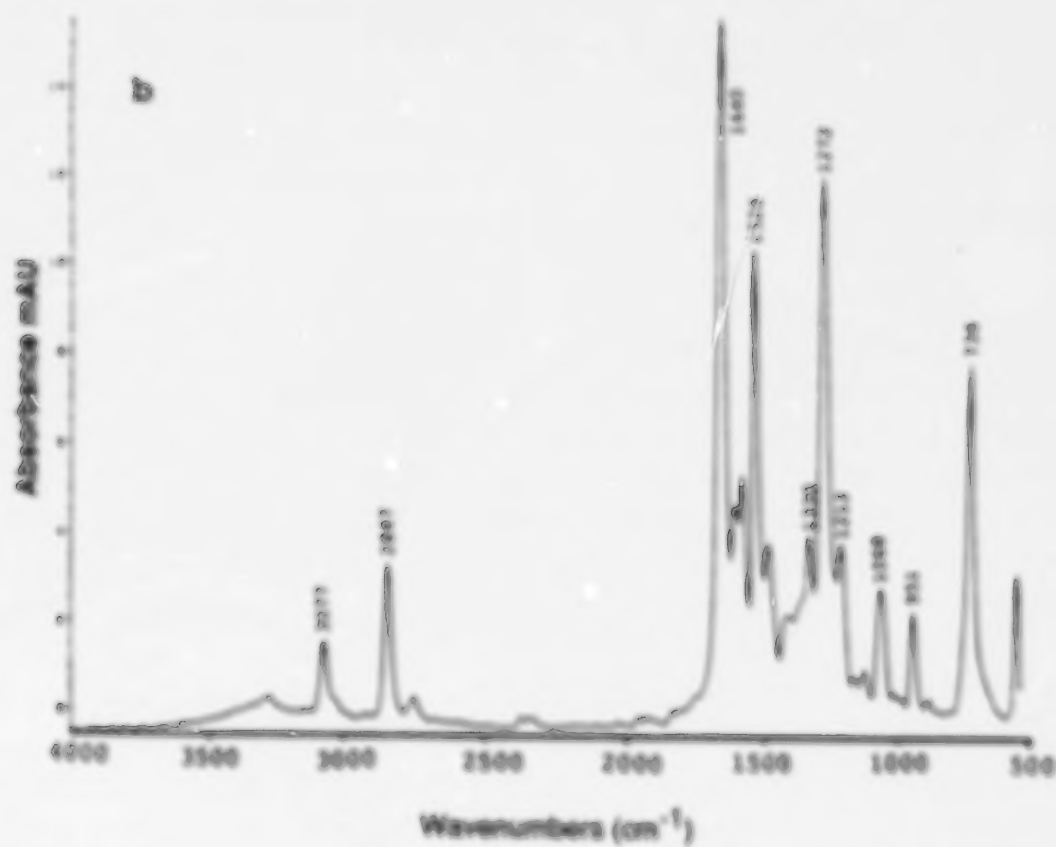
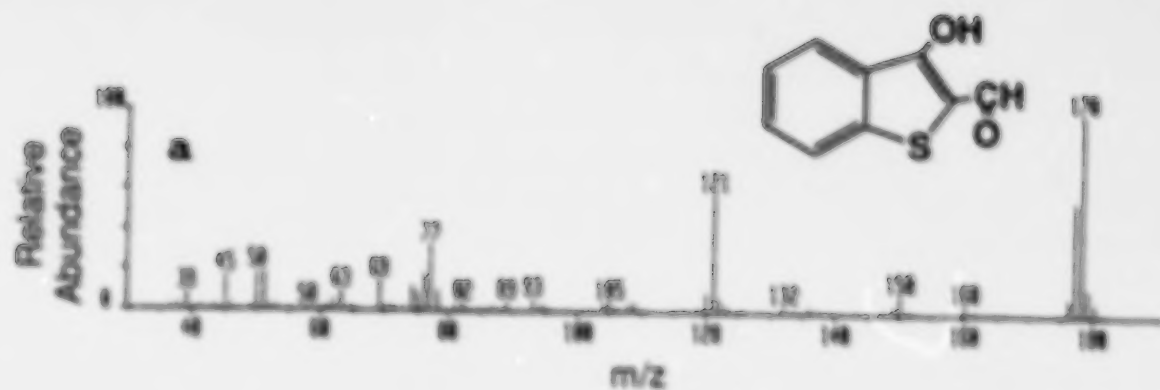


Figure S.1. Spectra of 10PBT: (a) mass spectrum (b) FTIR spectrum.

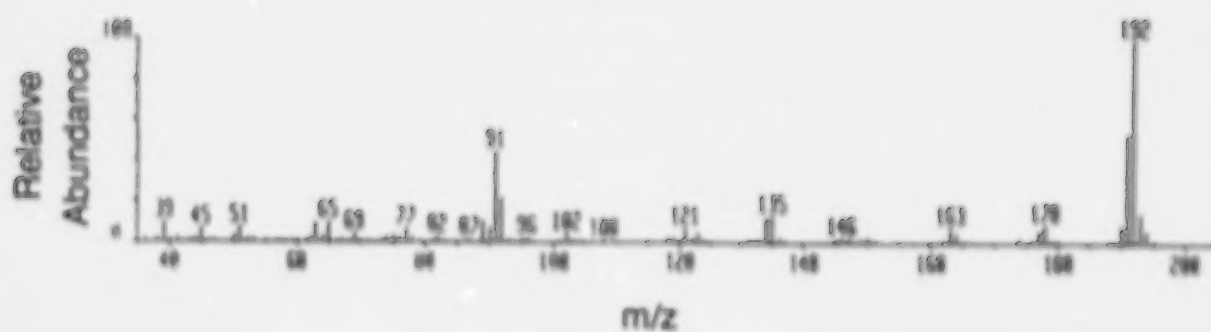


Figure 5.2. Mass spectrum of the metabolite from 4-methyldibenzothiophene, tentatively identified as 7-methyl-3-hydroxy-2-formylbenzothiophene by Fedorak (1990).

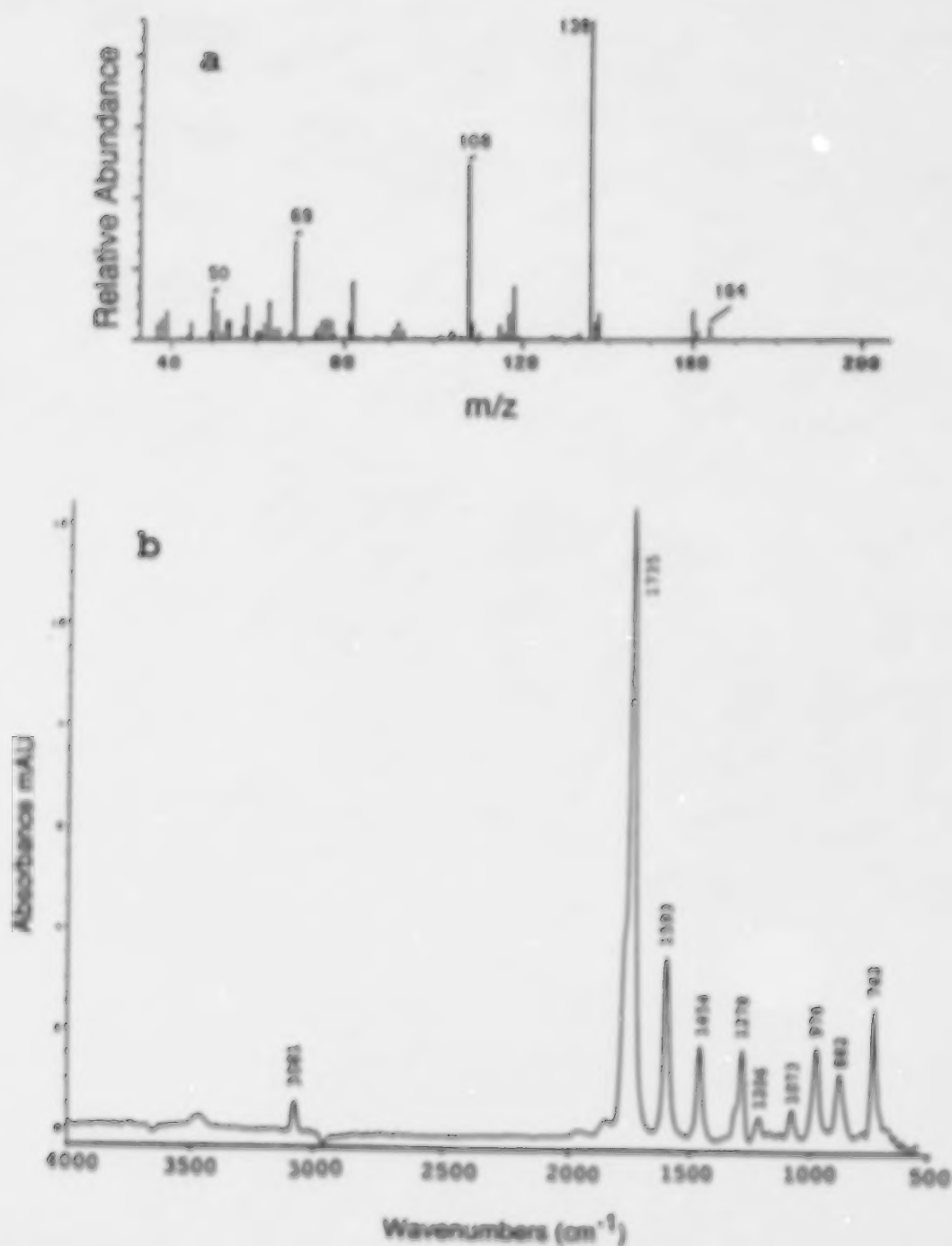


Figure 5.3. Spectra of the major metabolite detected in a culture of isolate BT1 grown on 1-MN in the presence of DBT; (a) mass spectrum (b) FTIR spectrum.

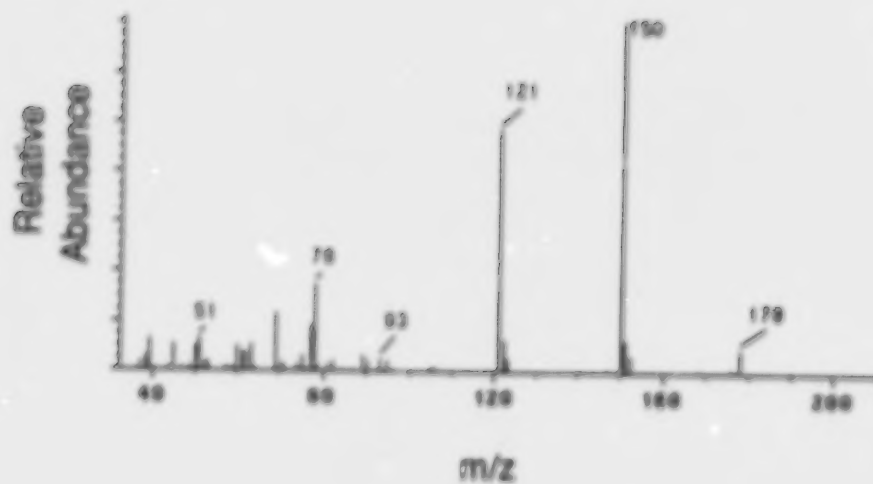


Figure S.4. Mass spectrum of the major metabolite detected in a culture of isolate BT1 grown on 1-MN in the presence of 2-methylbenzothiothiophene.

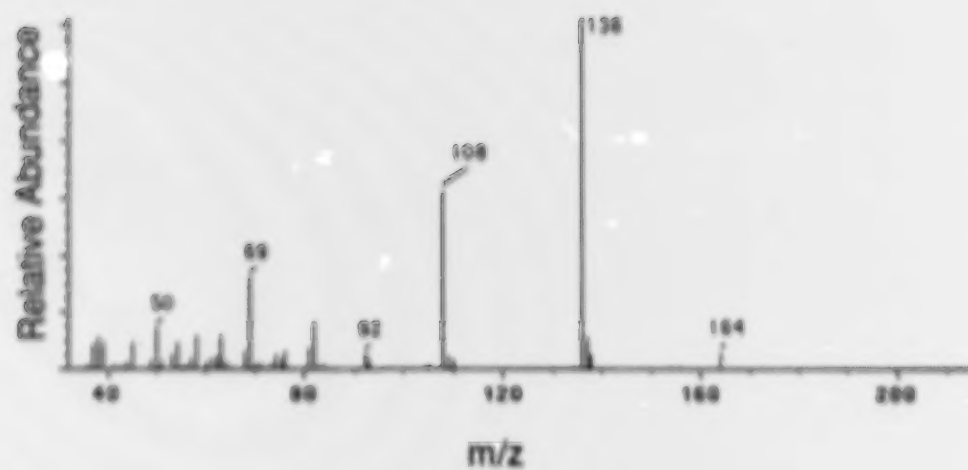


Figure S.5. Mass spectrum of the minor metabolite detected in a culture of isolate BT1 grown on 1-MN in the presence of 2-methylbenzothiophene.

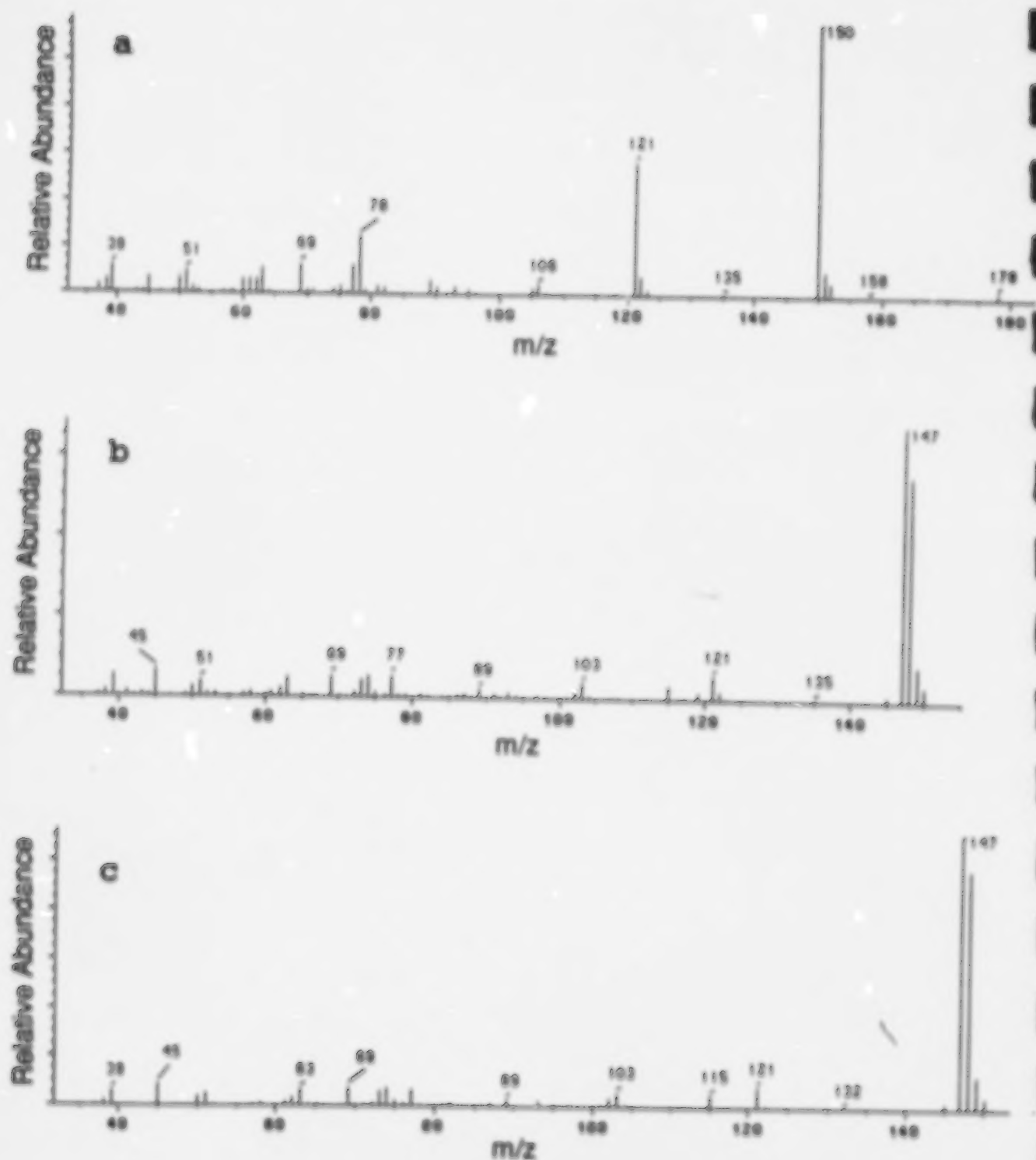


Figure 5.6. Mass spectra of (a) the dione, (b) the methylbenzothiophene detected in a culture of isolate BT1 grown on 1-MN in the presence of 3-methyldibenzothiophene, and (c) authentic 6-methylbenzothiophene.

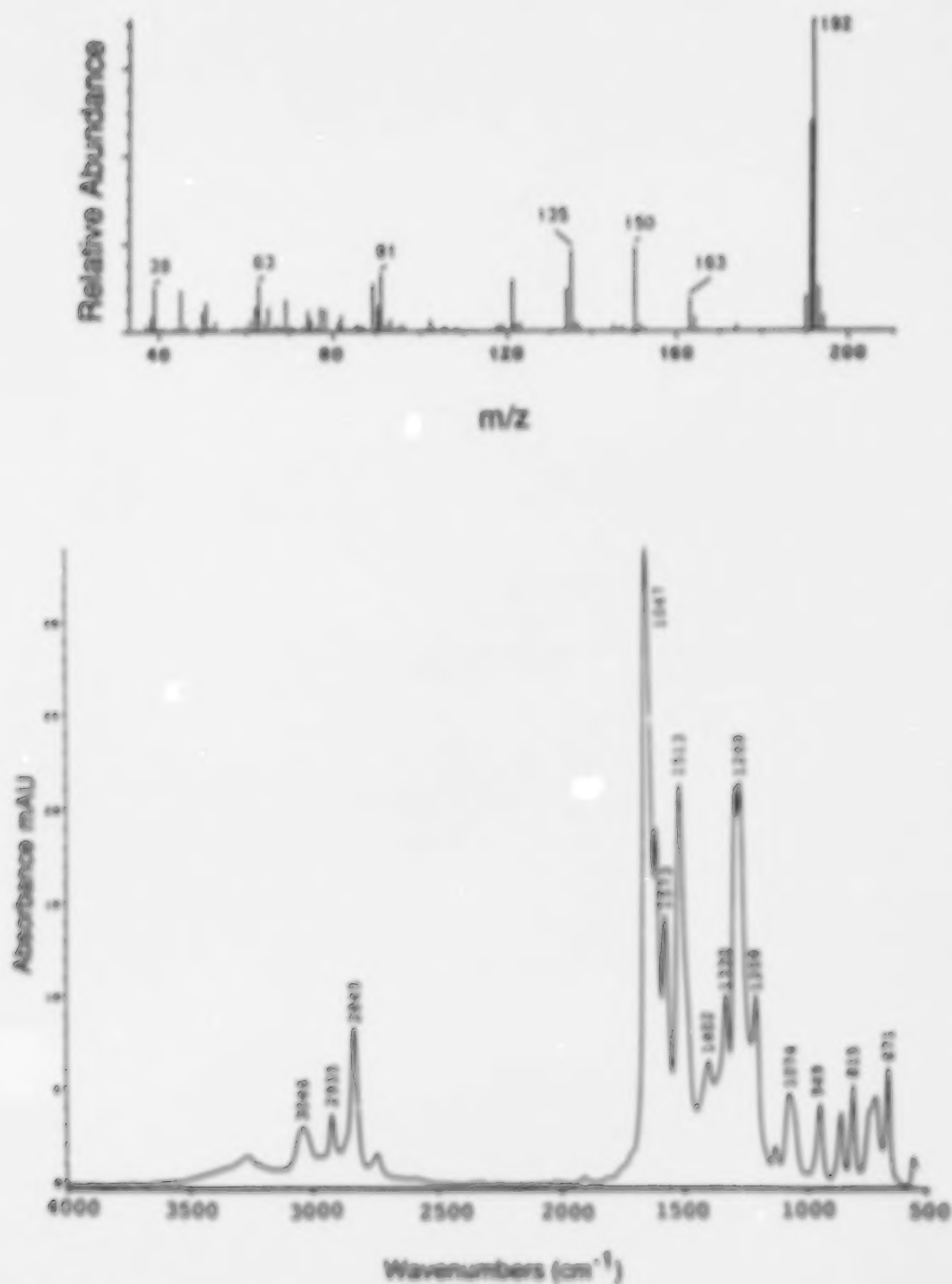


Figure 3.7. Spectra of the major metabolite detected in a culture of isolate BT1 incubated in the presence of 3-methylbenzothiophene; (a) mass spectrum, (b) FTIR spectrum. The inoculum used for this experiment had been maintained on DBT only.

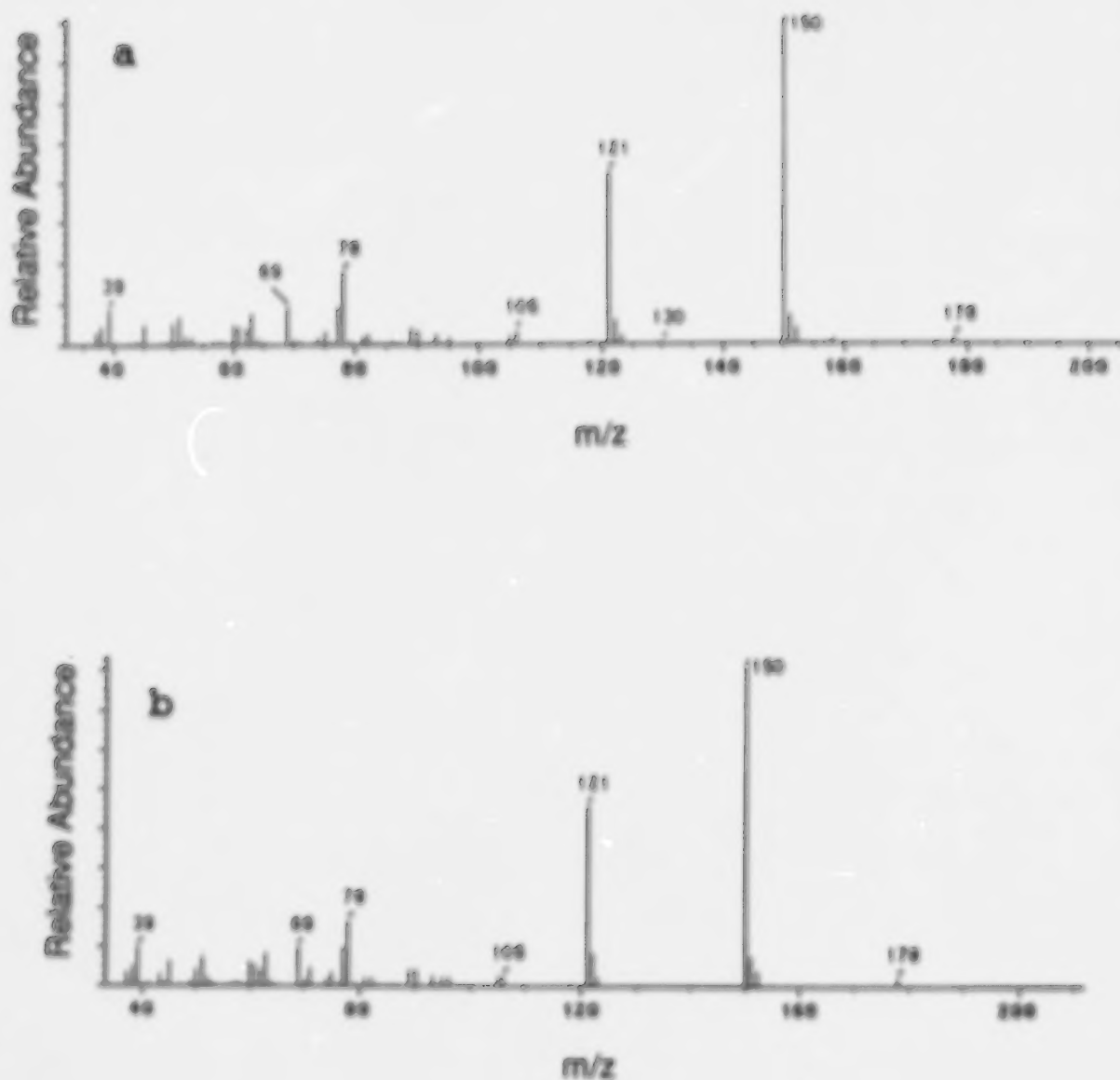


Figure 5.8. Mass spectra of the metabolites detected in a culture of isolate BT1 grown on 1-MN in the presence of a mixture of 1-methylbenzothioephene and 3-methylbenzothioephene; (a) metabolite with the same GC retention time as that from 3-methylbenzothioephene (Figure 5.6a), (b) metabolite presumed to be from 1-methylbenzothioephene.

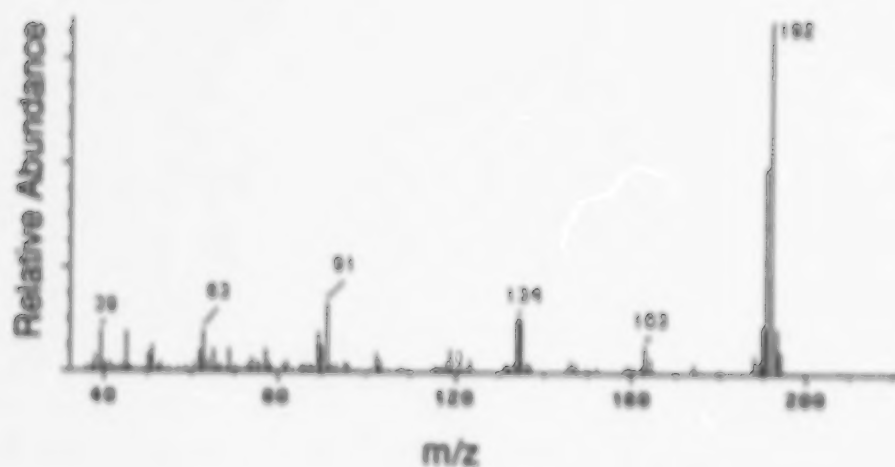


Figure S.9. Mass spectrum of the major metabolite detected in a culture of isolate BT1 grown for 2 days on 1-MN in the presence of 4-methylthiobenzoate.

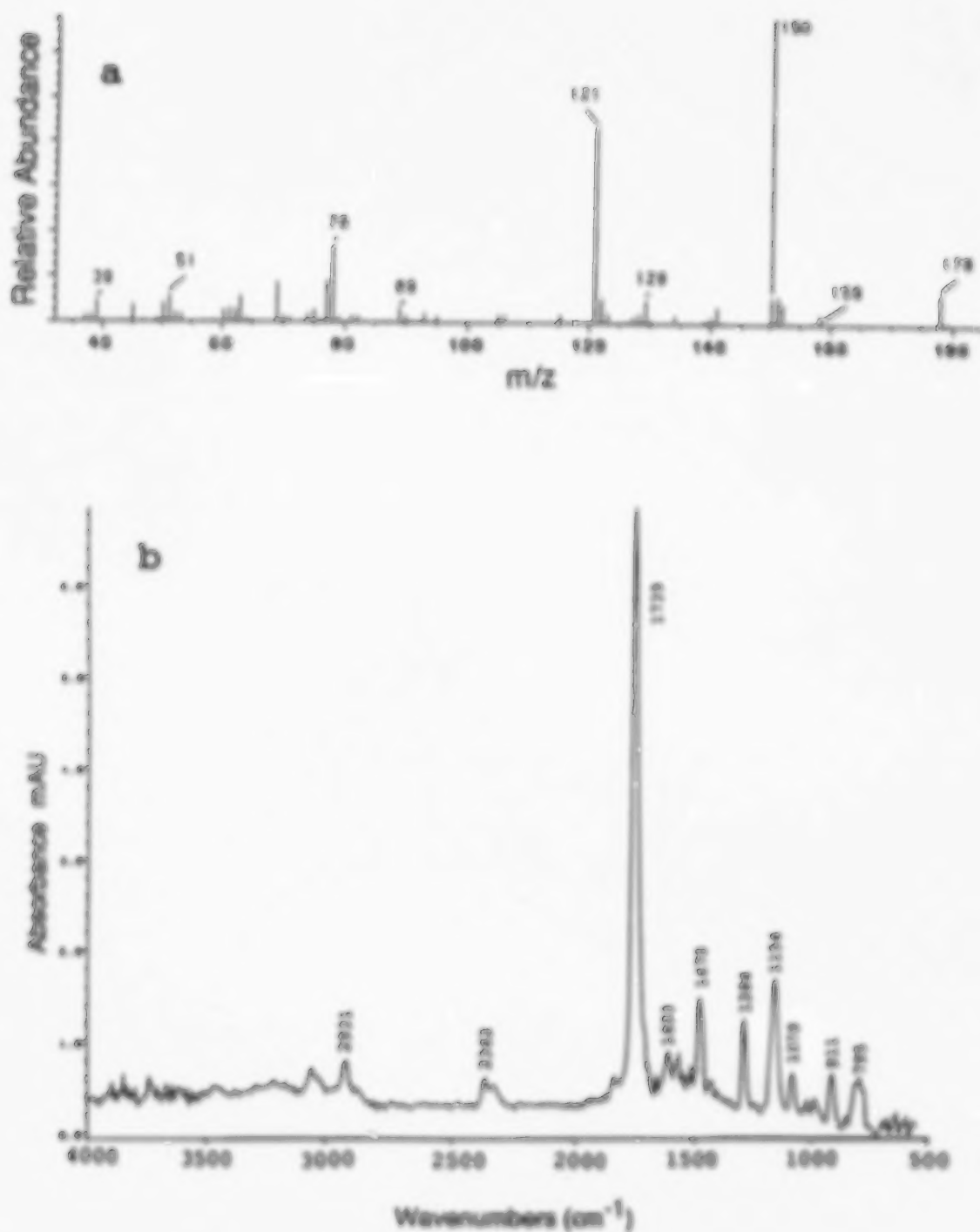


Figure 5.10. Spectra of a metabolite detected in a culture of isolate W1 grown on 1-MN in the presence of 2-methyldibenzothiophene; (a) mass spectrum, (b) FTIR spectrum.

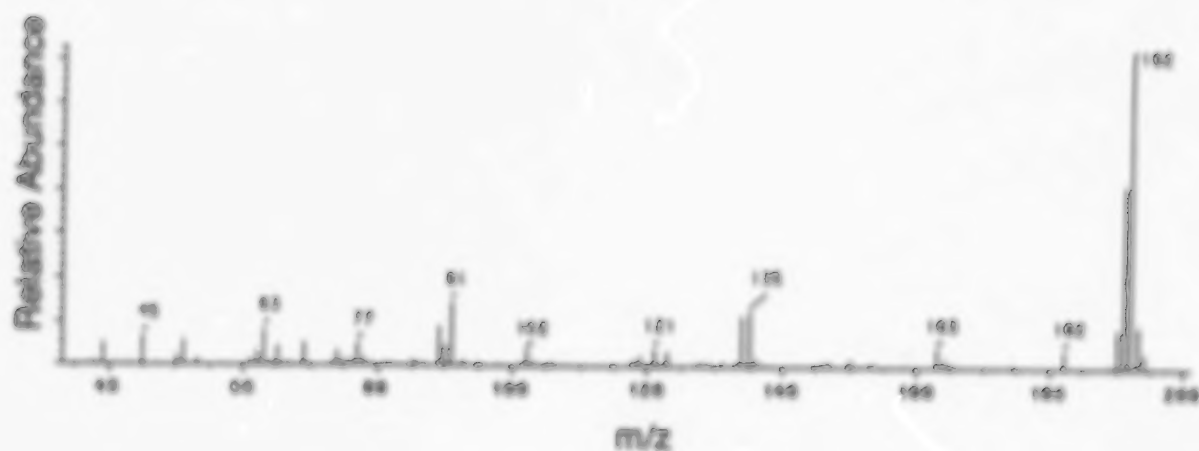


Figure S.11. Mass spectrum of the major metabolite obtained from a culture of isolate W1 grown on 1-MN in the presence of 4-methylbenzanthracene.

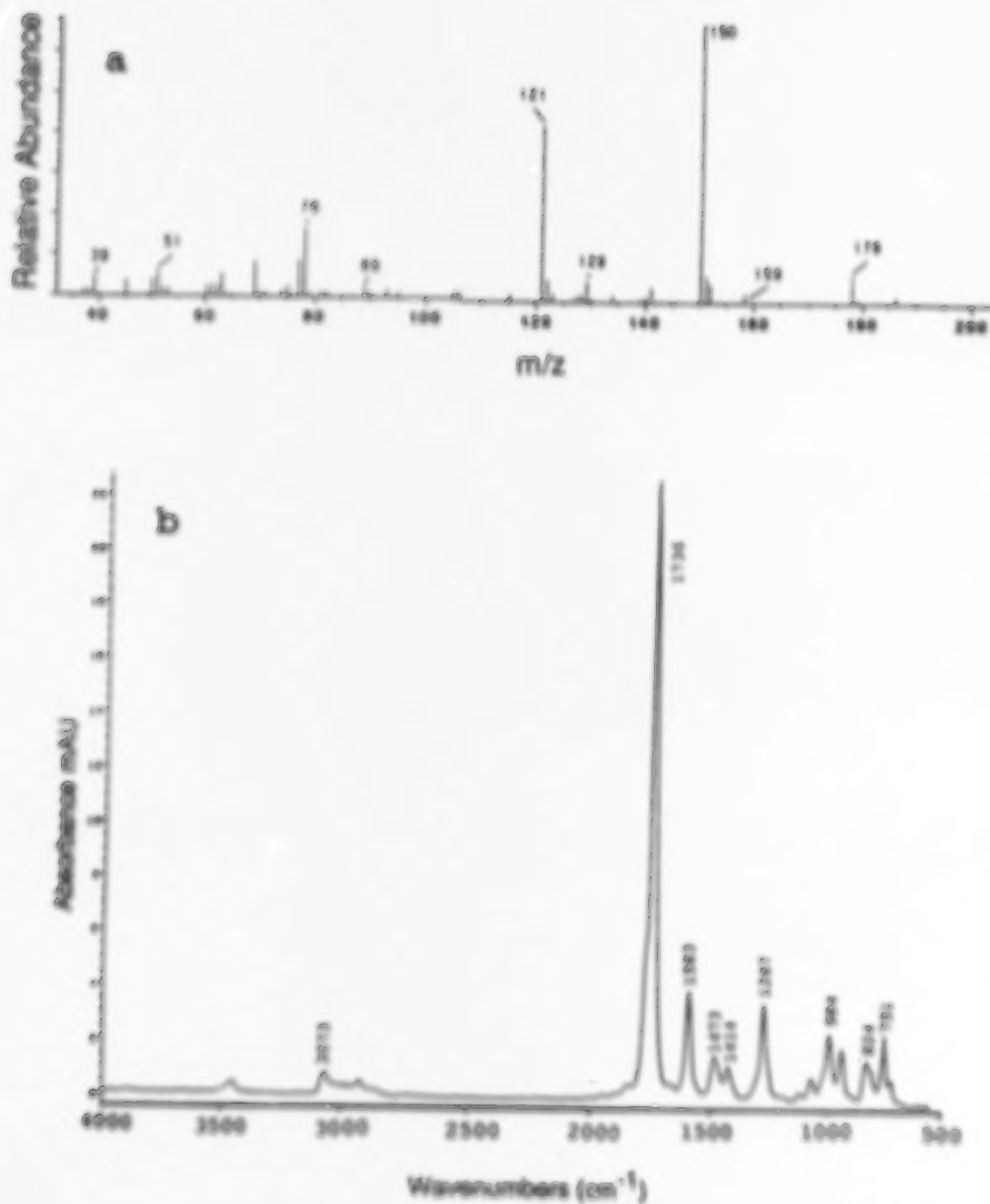


Figure 9.12. Spectra of the minor metabolite obtained from a culture of isolate W1 grown on 1-MN in the presence of 4-methylbiphenylsulfone; (a) mass spectrum, (b) FTIR spectrum.

6.0 ACKNOWLEDGMENTS

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Appendix: Presentations and publications arising from this project.

1. A poster entitled "Aerobic microbial cometabolism of methylbenzothiophenes" was presented in May, 1991 at the 91st Annual Meeting of the American Society for Microbiology in Dallas, Texas.
2. A paper entitled "Decomposition of two methylbenzothiophene sulfoxides in a commercial GC injection port liner" by P.M. Fedorak and J.T. Anderson was published in the *Journal of Chromatography*, Vol. 591, pp. 362-366, 1992.
3. A presentation entitled "Microbial degradation of some *n*-alkylthiophenes" by P.M. Fedorak and T.M. Priskman was given at the 1991 BIOMINET meeting in Calgary and at the Fourth International Symposium on Gas, Oil and Environmental Biotechnology in Colorado Springs. Written copies of these presentations will appear in the proceedings of both conferences.
4. P.M. Fedorak was invited to San Diego where he presented a seminar entitled "Petroleum biodegradation - twenty years of studies at the University of Alberta" at San Diego State University and at Scripps Institute of Oceanography. Approximately one-third of the material in these talks focused on work with organosulfur compounds.
5. An abstract entitled "Aerobic microbial cometabolism of methyl dibenzothiophenes by bacterial cultures" by P.M. Fedorak, S. Saffir and J.T. Anderson was accepted for presentation in May 1992 at the 92nd Annual Meeting of the American Society for Microbiology in May in New Orleans.
6. A manuscript entitled "Aerobic microbial metabolism of some alkylthiophenes found in petroleum" by P.M. Fedorak and T.M. Priskman was accepted for publication in *Biodegradation*.
7. A manuscript entitled "Diones, Sulfoxides and Sulfones from the Aerobic Cometabolism of Methylbenzothiophenes by *Pseudomonas* strain BT1," by S. Saffir and P.M. Fedorak and J.T. Anderson has been submitted to *Environmental Science & Technology* for review.
8. P.M. Fedorak was invited to present a paper at the 1992 meeting of the Canadian Chemical Conference & Exhibition in June in Edmonton. An abstract entitled "Aerobic microbial metabolism of alkyl and combined thiophenes" by P.M. Fedorak, J.T. Anderson and T.M. Priskman has been accepted for presentation.
9. The data gathered on the metabolism of the alkylbenzothiophenes and alkyl dibenzothiophenes will form the basis for the PhD thesis to be written by S. Saffir for submission to the University of Essen in Germany.